



Genetically Engineered Human Kidney Cells for Real-Time Cytotoxicity Testing In Vitro

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Abstract

Classic toxicology studies often utilize in vivo animal models. Newer approaches employing in vitro organ-specific cellular models have been developed in recent years to help accelerate the speed and reduce the cost of traditional toxicology testing. Toward the goal of supporting in vitro cellular model research with a regulatory application in mind, we have developed a ‘designer’ human kidney cell line called HK2-Vi that can fluorescently measure the cytotoxicity of potential toxins on proximal tubule cell viability in a direct exposure in vitro model. HK2-Vi was designed to be a reagent-less kinetic assay that can yield data on short- or long-term cell viability after toxin exposure. To generate HK2-Vi, we used monocistronic lentiviral transduction methods to genetically engineer a human kidney cell line called HK-2 to stably co-express two transgenes. The first is Perceval HR, which encodes a fluorescent biosensor of both cytosolic ATP and ADP and the second is pHRed, which encodes a biosensor of cytosolic pH. Relative levels of cellular ATP and ADP effectively serve as a reliable and robust indicator of cell viability. Because the fluorescence Perceval HR is pH-dependent, we co-expressed the pHRed genetic biosensor to correct for variations in pH if necessary. Heterogenous populations of transduced renal cells were enriched by flow cytometry before monoclonal cellular populations were isolated by cell culture methods. A single clonal population of co-transduced cells expressing both Perceval HR and pHRed was selected to be HK2-Vi. This established cell line can now serve as a tool for in vitro toxicology testing and the methods described herein serve as a model for developing designer cell lines derived from other organs.

Keywords Biosensor · ATP · ADP · pH · Kidney proximal tubule · Nephrotoxicity

Introduction

The regulation of foods, drugs, biologicals, and other industries is historically guided by classic toxicology research studies utilizing in vivo animal models. A gradual decline in the use of animal model testing has gained momentum in part due to the acceptance of the ‘Replacement, Reduction,

and Refinement’ (RRR) concept, which was first introduced in 1959 [1]. This concept promotes the use of alternative assays to decrease the need for animal testing. To date, a few in vitro techniques for chemical testing have been accepted by the Organization for Economic Co-operation and Development (OECD) and they have allowed many countries to ban cosmetics testing using animal models, for example [2, 3]. In vitro testing has also proven helpful in predicting animal and human outcomes as well as addressing mechanistic aspects of cellular function across many disciplines [4].

As part of an ongoing effort to improve in vitro cellular toxicity testing, we have developed a prototype method to create a cell line that can be used to quantitatively measure cell viability in an experimental model of both short- and long-term direct toxin exposure(s). Since current in vitro cell viability testing methods mostly rely on commercially developed kit assays [5], we were motivated to develop more efficient ways to study cell viability during in vitro cellular toxicity testing experiments. We were also interested in

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circumventing the use of commercial cell viability assays because the reagents (fluorescent dyes, for example) that they require render them as terminal (or endpoint) assays; once the viability of a cell population has been evaluated, the same cell population cannot be re-evaluated at a later time. These aspects clearly hamper efforts to scale up the throughput of cytotoxicity experiments, especially when multiple exposure doses and exposure times need to be tested. As such, we turned to the gene transfer biotechnology field to genetically engineer a cell line of choice to express a recently developed genetic biosensor of cell viability called Perceval HR [6].

Genetic biosensors are DNA-encoded proteins that are designed to bind to specific molecules and induce a measurable change that can be used to monitor frequency or strength of target binding. Typical biosensors indicate target-sensing via allosteric changes in their structure that translate into changes in fluorescence emission. Examples include biosensors of calcium [7] or magnesium [8] ions, glucose [9] or glutamate [10] transport across a cell membrane, intracellular caspase activity [11], intracellular ATP and ADP levels [6], and pH levels [12]. Since ATP is a fundamental biomarker of cell viability [13, 14], we selected the biosensor Perceval HR, which simultaneously detects cellular ATP and ADP. Perceval HR is a fusion protein between a bacterial regulatory protein, GlnK1, and a modified green fluorescent protein (GFP). As this biosensor binds to ATP or ADP, its 3D protein structure changes to produce a change in fluorescence emission that is detectable by standard fluorescent plate readers. Like many fluorescent biosensors, the pH inside cells can affect the 3D structure of proteins and hence affect the fluorescent readings. Taking this physical property into account, Perceval HR was designed to be used in tandem with an intracellular pH biosensor called pHRed [12], which we have also adopted.

In developing a method to incorporate both Perceval HR and pHRed biosensors into a target cell line, we selected the human kidney HK-2 cell line because the kidney is among the most susceptible target organs of toxicity [15] and this cell line is widely used in studies of in vitro toxicology [16–19]. The overall schema to develop this method consisted of (a) obtaining the DNA encoding Perceval HR and pHRed on a lentiviral backbone, (b) transfecting this DNA into a producer line (293 T cells) to generate replication-deficient virions that have the ability to stably integrate into the genome of the host cell and constitutively express payload genes, and (c) collect these virions encoding Perceval HR and pHRed to stably transduce HK-2 cells and isolate the final monoclonal cell line product we coined HK2-Vi.

Methods

Plasmid Stocks

FUG-W-pHRed DNA transfer plasmid was received as a gift from Dr. Tantama (Purdue University). A glycerol stock of *E. coli* transformed with the Perceval HR DNA transfer plasmid, FUG-W-Perceval HR, was purchased from Addgene (Cambridge, MA). Bacterial agar stabs carrying the accessory plasmids required for packaging and enveloping the virions, psPAX2 and pMD2.G, were also purchased from Addgene. We prepared a pHRed bacterial glycerol stock by transforming One Shot Stbl3 *E. coli* cells (Thermo Fisher, Waltham, MA) with FUG-W-pHRed DNA, following the manufacturer's instructions. All glycerol stocks were then used to inoculate LB Broth (Sigma, St. Louis, MO) cultures grown with 100 µg/ml of ampicillin (Sigma) for about 15 h at 37 °C with a shaking incubator set at 225 rpm. DNA plasmids were extracted from these cultures and purified using the Endotoxin-free Maxiprep kit (Sigma). Their quantity and purity were measured with a Nanodrop spectrophotometer (Thermo Fisher). Plasmid identities were confirmed by 0.8% agarose gel electrophoresis using the E-Gel-iBase system (Thermo Fisher) following standard overnight restriction enzyme digestion by EcoRI (10 units/µg DNA; New England Biolabs, Ipswich, MA).

DNA Transfections

The packaging cell line Lenti-X 293T cells (Clontech, Mountain View, CA) was transfected with the transfer and accessory DNA plasmids needed to produce replication incompetent virions. Cells were plated at 70% confluency in 15-cm tissue culture-treated plates (VWR, Radnor, PA) in DMEM (Sigma) supplemented with Heat-inactivated FBS and L-glutamine (Sigma) overnight at 37 °C/5%CO₂. For each payload gene, a separate master plasmid mixture was prepared; each mixture consisted of lentiviral transfer vector (23.0 µg), packaging vector (11.5 µg) and envelope vector (11.5 µg). All master mix and vector solutions were prepared in polypropylene tubes in DMEM (serum-free) and then 115 µl of Fugene6 (Promega, Madison, WI) were directly added to each master mixture. Final mixtures were gently vortexed, incubated at room temperature for 20 min, and added dropwise to the 293T cells in their 15-cm plates. Transfected cells were kept overnight at 37 °C/5%CO₂ and then their media was gently removed and replaced with Keratinocyte SFM media (Thermo Fisher) supplemented with 5% HI FBS. Cells were re-incubated for 24 h to give the cells time to produce and release virions into their

supernatants, which were collected by pipette and filtered by 0.45 nm syringe filter to remove cellular debris.

Human Proximal Tubule Cell Line Transduction

To transduce the human kidney target cells, HK-2 (ATCC, Manassas, VA) to express both Perceval HR and pHRed, approximately 5 ml of filtered viral supernatant from 293T cells transfected with each of these two payload genes were mixed with 10 µl of protamine sulfate 4 mg/ml stock solution prepared in sterile water (Sigma). All 10 ml of supernatants were then overlaid onto HK-2 cells that had been seeded in T25 flasks (VWR) the day before at approximately 20% confluence. As experimental controls, we also singly transduced HK-2 cells Perceval HR or pHRed alone. Transduced HK-2 cells were incubated for 48 h at 37 °C/5%CO₂ before replacing their viral supernatant media to fresh Keratinocyte SFM media containing 5% HI FBS and allowed to expand to confluency.

Single Cell Clone Establishment

Expanded transduced HK-2 cells were checked for transgene expression using a FACS Aria flow cytometer (BD Biosciences, La Jolla, CA) since the absorption and emission profiles of Perceval HR and pHRed were designed to be spectrally non-overlapping and can be viewed with minimal color compensation on the FITC and PerCP-Cy5.5 fluorescence channels, respectively. Expression of all payload genes was verified in the transduced HK-2 cells before monoclonal cell lines from these heterogeneous populations were prepared in two steps. First, cell sorting was performed by FACS Aria to enrich each population for the top 50–70% most brightly fluorescing cells. Once the enriched cells had recovered from their sorting procedure and could be carefully passaged, the second step was to harvest and seed them at the very low density of 1 cell per ml of culture media in 96-well plates. After approximately 2 weeks, colonies visible to the naked eye appeared in these plates and were sequentially expanded into 24- and 6-well plates before being transferred to T75 flasks for routine maintenance. These final cultures were checked by flow cytometry for their clonal expansion to select the highest expressing clone, HK2-Vi.

Cytotoxicity Testing

Parental HK-2 and transduced HK2-Vi cell lines were treated for 24 h with several compounds known to either induce toxicity or not. HK-2 cells were tested for cytotoxicity using ATP level measurements from the CellTiter-Glo Cell Viability Assay kit from Promega (Madison, WI).

Table 1 List of test compounds used to compare the parental HK-2 cell line to its modified HK2-Vi version

Toxic compounds		References
1	Ochratoxin A	[20, 21]
2	Camptothecin	[22, 23]
3	Lapatinib	[24, 25]
4	Afatinib	[24]
5	Methotrexate	[26–28]
6	Citrinin	[20, 29]
7	Diaminophenol	[30, 31]
8	Doxorubicin	[22, 29]
9	Vancomycin	[32]
10	Idarubicin	[33, 34]
Innocuous compounds		
1	Dexamethasone	[35]
2	Allopurinol	[36, 37]
3	Methylparaben	[38]
4	Pioglitazone	[39]
5	Atenolol	[38, 40]
6	Acarbose	[41, 42]
7	Sodium diatrizoate	[43]
8	Trichloroethylene	[44, 45]
9	Glycine	[46, 47]
10	Levodopa	[48, 49]

Small samples of twenty chemical compound solutions in DMSO (Enzo Life Sciences, Inc.; Farmingdale, NY) were serially diluted from 10 mM in HK-2 cell culture media by 10²- to 10⁶-fold levels (Table 1). The compounds were ochratoxin A, camptothecin, lapatinib, afatinib, methotrexate, citrinin, diaminophenol, doxorubicin, vancomycin, and idarubicin, dexamethasone, allopurinol, methylparaben, pioglitazone, atenolol, acarbose, sodium diatrizoate, trichloroethylene, glycine, and levodopa. Cells were seeded at 2 × 10⁴ cells/well in 96-well plates the day before they were treated at different concentrations of toxins before adding the luminescent Promega substrate and following the manufacturer's instructions to enzymatically detect ATP levels in each well. A standard plate reader (BMG LabTech; Cary, NC) was used to measure relative levels of ATP in replicates of three in luminescent mode for HK-2 cells and in fluorescent mode for HK2-Vi cells. The fluorescence values at wavelengths 695 and 520 nm were calculated to determine the ratio of ATP- to ADP-Perceval HR complexes. Fluorescence values for pHRed were measured at 610 nm to ensure that the cell culture media buffering capacity of chemical treatments were not exceeded. Two-way ANOVA tests were performed using the statistical Graph Pad Prism software (GraphPad; La Jolla, CA).

Results

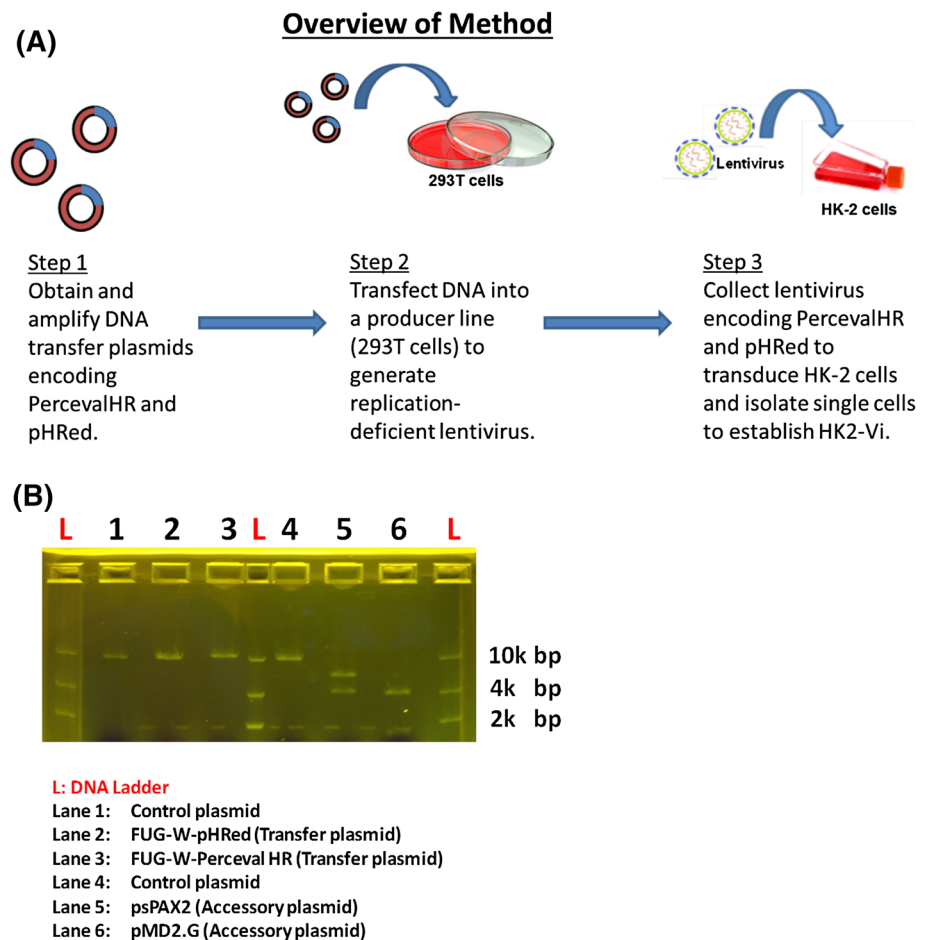
We set out to genetically engineer the HK-2 cell line, which is a well-established in vitro model of human kidney cells derived from proximal tubules, into a novel tool for rapid in vitro cellular toxicology testing. This transformation process was performed in the three simple steps presented in Fig. 1a. Naked DNA transfer plasmids encoding the transgenes of interest, Perceval HR and pHRed, were transfected into the 293 T cell producer line, which in turn budded virions that could be harvested for subsequent transduction of the HK-2 cells. To verify that the used transfer plasmids were of correct identities, EcoRI restriction enzyme digestion techniques were used. As shown in Fig. 1b, the correct band sizes of circular plasmids and their digested linear products were obtained. Transfer plasmids FUG-W-pHRed and FUG-W-Perceval HR were linearized into bands of correct size, approximately 9.9 kb and 10.9 kb, respectively. The packaging accessory plasmid psPAX2 was correctly digested into fragments of 6329 and 4374 bp. The envelope accessory

plasmid pMD2.G was also correctly linearized into fragments of 4153 and 1671 bp (Fig. 1b).

Once HK-2 cells were transduced with the appropriate pools of viral particles, flow cytometry was used to enrich the resulting heterogeneous pools of cells for transduced populations before single cell cloning was performed. The final single cell clones were screened by flow cytometry and the highest transgene-expressing clones were analyzed by flow cytometry to verify the clonal nature of the expanded populations and the expression levels of each transgene. As shown in Fig. 2, the single cell isolation method applied to each transduced population yielded monoclonal populations of single histogram peaks having 99.0% purity, concluding the generation phase of our newly coined HK2-Vi to represent the viability read-out that this cell line can provide.

To perform proof-of-principle experiments that demonstrate the utility of this newly modified cell line, we selected 10 toxins that are known to cause injury to proximal tubule cells and another 10 compounds that are considered innocuous to kidney cells (Table 1) [20–49]. In side-by-side experiments, parental cell line HK-2 and the newly made cell line HK2-Vi were exposed to increasing dilutions of 10 mM stock compounds ranging from 100× to 1,000,000×

Fig. 1 **a** Schematic diagram of steps in the method to develop HK2-Vi cells. **b** Confirmation of lentiviral vector DNA plasmid identities by restriction enzyme digestion with EcoRI



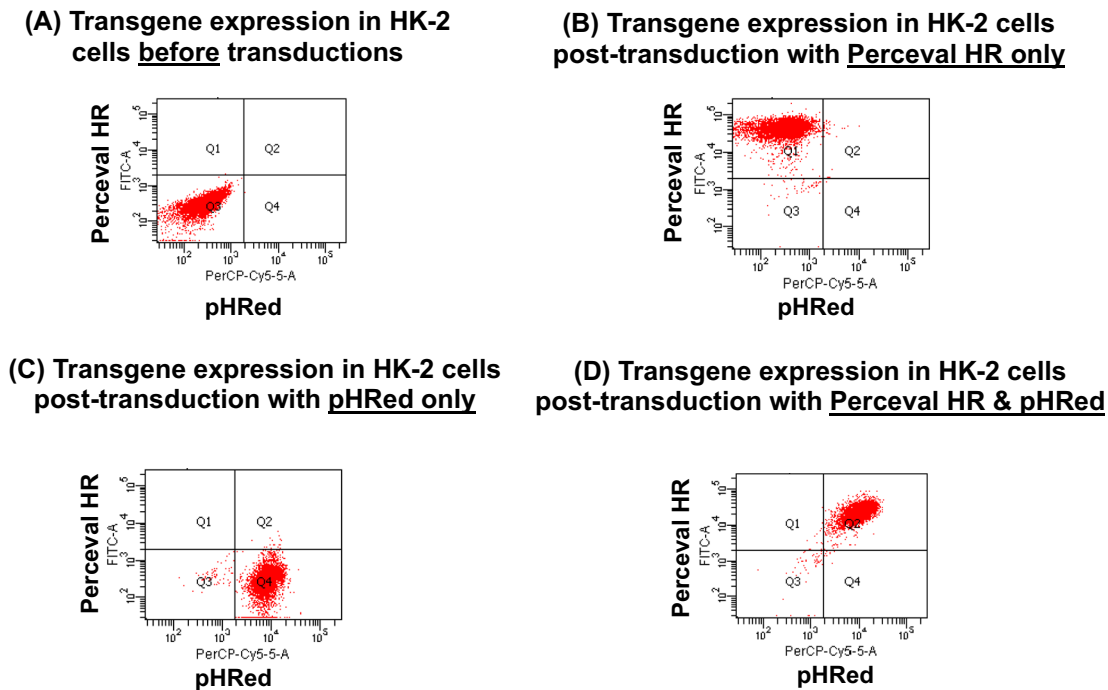


Fig. 2 Flow cytometric representation by histogram and dotplot graphs of the expression of fluorescent biosensor proteins. **a** Control parental HK-2 cell line lacks inherent fluorescence. **b** HK-2 cells transduced with the single transgene Perceval HR and grown from a single cell exhibits fluorescence exclusively ($\geq 99\%$) in the FITC channel. **c** HK-2 cells transduced with the other biosensor gene,

pHRed, and grown from a single cell fluoresces exclusively in the PerCP-Cy5.5 channel ($\geq 99\%$). **d** Co-transduction of HK-2 cells with the two biosensor transgenes, Perceval HR and pHRed, followed by single cell isolation generated Cell-Vi, whose fluorescence expression in both channels is $\geq 99\%$

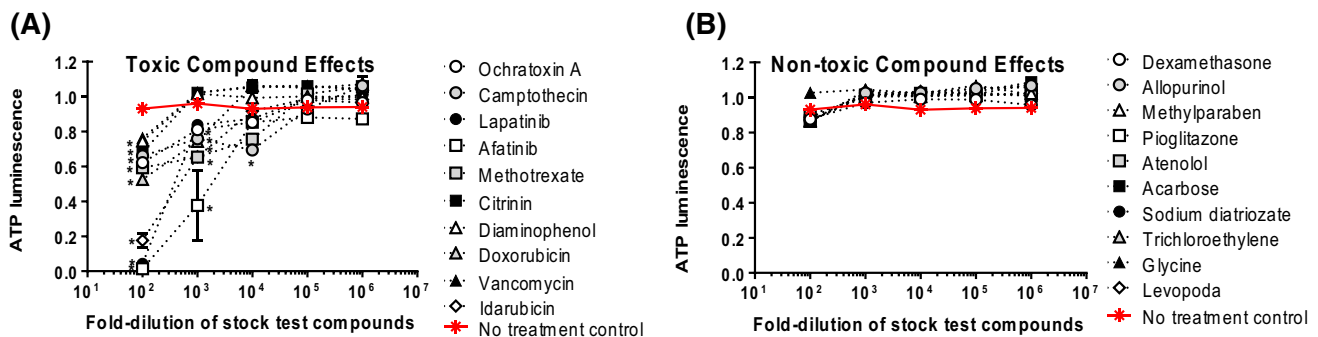


Fig. 3 Cytotoxicity testing of **a** 10 nephrotoxins and **b** 10 non-toxic compounds toward human proximal tubule cell line, HK-2, using a commercial luminescence assay for cellular ATP measurement.

dilutions. As shown in Fig. 3a, high doses of all tested nephrotoxins compromised cell viability in HK-2 cells, as measured by relative cellular ATP content. With increasing dilutions, the dose–response effect yielded more ATP and greater cell viability. By contrast, the innocuous compounds had little to no effect in the same concentration range tested (Fig. 3b). Interestingly, we found that only seven out of 10 toxins reduced the ratio of ATP to ADP when the Perceval

Graphs represent the mean average of three experimental tests. Significant differences were determined with *P* values of less than 0.05

HR/pHRed-expressing HK2-Vi cells were tested under the same experimental conditions as the HK-2 cells. As shown in Fig. 4a, three compounds, doxorubicin, vancomycin, and idarubicin, actually yielded false increases in cell viability in a dose-dependent manner. Similarly, they produced anomalous effects in the pHRed fluorescence (Fig. 4c), whereas all other tested compounds yielded stable pHRed signals (Fig. 4c, d). Clearly, doxorubicin, vancomycin, and

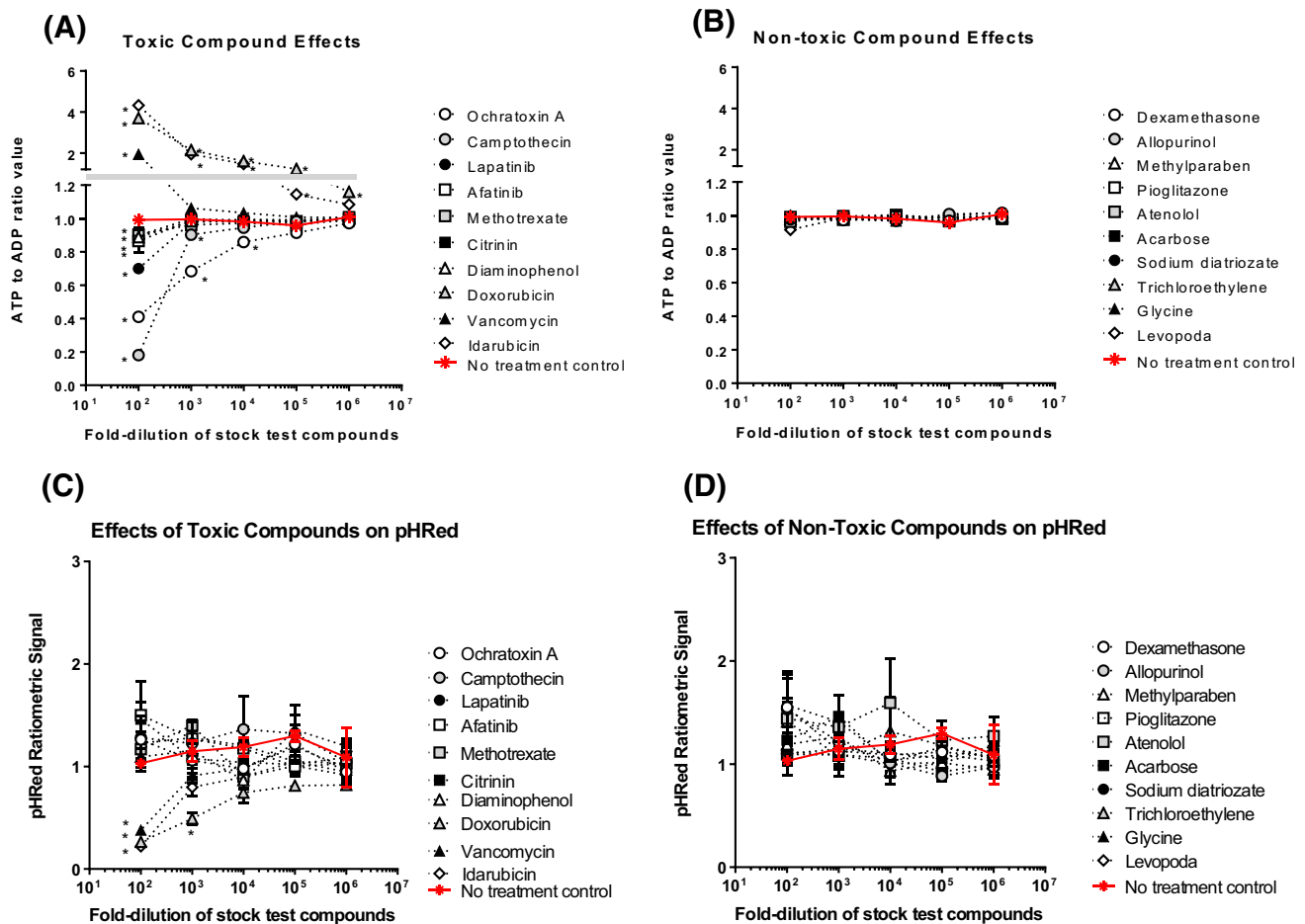


Fig. 4 Fluorescence assay of cellular ATP levels relative to ADP levels in HK2-Vi cells using **a** 10 nephrotoxins and **b** 10 compounds that are considered to be non-nephrotoxic. Corresponding pHRed signals for each toxic (**c**) and non-toxic (**d**) compound are also shown

idarubicin uniquely possess inherent fluorescence that overlaps with the spectra of HK2-Vi cells. Thus, pre-screening compounds of interest for intrinsic fluorescence in the 520 nm or 695 nm emission wavelength areas should be performed prior to using HK2-Vi cells for in vitro cytotoxicity testing to avoid artefactual results. Such naturally fluorescing compounds are not compatible with this cell line tool. Despite this limitation, none of the innocuous compounds tested for toxicity yielded false positive results (Fig. 4b).

Our method of developing HK2-Vi as a tool to evaluate the potential toxicity of chemicals of interest relies on standard genetic engineering methods that combine aspects of microbiology as well as molecular and cellular biology laboratory techniques. This modified human proximal tubule cell line has value in rapidly and inexpensively screening compounds of interest, provided that their fluorescence spectra profiles do not overlap with green and red wavelength ranges. Nevertheless, the field of in vitro cellular toxicology would benefit from our developing

for each tested concentration. Graphs represent the mean average of three experimental tests. Significant differences were determined with *P* values of less than 0.05

this tool for preliminary testing purposes for two main reasons. First, it would significantly speed up the ability of research scientists interested in basic and regulatory toxicology testing to quantitatively assess the impact of directly exposing human kidney cells to one or more chemicals in real-time. Second, the application of lentiviral vectors to carry out the transduction steps used to create HK2-Vi means that it can easily be applied to create other cell lines derived from organs from any species to stably express other genetic biosensors and test a variety of compounds. Overall, the simplicity of this cell line tool as well as its method of creation together underscore the utility and versatility of its application. Further testing beyond proof-of-principle studies will be required to comprehensively validate its application in a range of settings to better understand its strengths and limitations of use.

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