Toxicology in Vitro 29 (2015) 1759–1765

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Improved assays for xenosensor activation based on reverse transfection

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ARTICLE INFO

Article history:
Received 4 April 2015
Revised 4 June 2015
Accepted 13 July 2015
Available online 14 July 2015

Keywords:
Aryl hydrocarbon receptor
Constitutive androstane receptor
Pregnane X receptor
Induction
Cytochrome P450
Reverse transfection

ABSTRACT

Discovery of receptor-dependent mechanisms for regulation of drug metabolism has provided a new way to evaluate the propensity of drug candidates to cause induction of cytochrome P450 enzymes. Therefore, receptor-based reporter assays have become common in early stages of drug development projects and in mechanistic studies. Here, we report a reverse transfection system to conduct activation assays for human xenosensors AhR, CAR and PXR. The assay format is based on long-term stability and uniformity of DNA/carrier complexes on culture plates, avoiding multiple stages and variation inherent in conventional transfection methods. Consequently, these improved assays are streamlined, reproducible and formally validated with Z' factors exceeding 0.5. This novel reverse transfection system is expected to find use in diverse areas of early drug development such prediction of CYP induction, evaluation of species differences and in mechanistic studies.

1. Introduction

The evaluation of induction of cytochrome P450 enzymes (CYPs) is an important step in drug development projects (Walsky and Boldt, 2009), because induction can cause long-lasting increases in drug metabolism, difficulties in selecting proper drug dosage, decreased therapeutic efficacy, increased toxicity due to enhanced production of reactive metabolites and disturbances of hormonal homeostasis (Pelkonen et al., 2008). The evaluation of CYP induction is usually conducted by measuring the key CYP1A2, CYP2B6 and CYP3A4 enzyme activities and mRNAs in human primary hepatocytes (HPHs) after exposing them to vehicle, test chemicals and positive control compounds such as omeprazole (OME), phenobarbital (PB) and rifampicin (RIF) (Hewitt et al., 2007; FDA, 2012). Elucidation of mechanisms for CYP induction (Pelkonen et al., 2008; Xie, 2009) have established that human aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR) control induction of these key CYP induction markers, respectively (FDA, 2012). Therefore, receptor-based *in vitro* assays have gained increasing use in early stages of drug development and in mechanistic studies (Stanley et al., 2006; Sinz et al., 2009). Although stable cell lines provide an easy-to-use and preferred source for long-term use, they are only available for human AhR and PXR (Raucy and Lasker, 2013). In studies involving multiple receptors and different reporter genes, generation and characterization of each individual cell subline becomes an arduous task. In turn, transient systems often utilize different cell lines, comprise of diverse reporter and expression systems, and diverge in concentrations and nature of the positive control compounds used. Thus, comparisons between assays become quite difficult. Furthermore, the assays have been formally validated only in few reports (*e.g.* Cui et al., 2008; He et al., 2011). In addition, the high basal activity of CAR in transfected cells has often complicated the assay development (Molnár et al., 2013).

Recently, we have optimized and formally validated transient transfection assays for all three human xenosensors AhR, CAR, and PXR (Küblbeck et al., 2011a). This assay requires separate stages for DNA/carrier complex formation, transfection of DNA into cells, removal of the DNA/carrier-containing medium and exposure to test chemicals. These stages collectively add to workload and sources for variability. In order to streamline these xenosensor

Abbreviations: AhR, human aryl hydrocarbon receptor; BNF, β-naphthoflavone; CAR, human constitutive androstane receptor; CV, coefficient of variation; CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; FDA, Food and Drug Administration; FL81, 5-(3,4-dimethoxybenzyl)-3-phenyl-4,5-dihydroisoxazole; FL82, 5-benzyl-3-phenyl-4,5-dihydroisoxazole; HPH, human primary hepatocytes; NIH, National Institutes of Health; OME, omeprazole; PB, phenobarbital; PEI25, polyethylene imine 25; PK11195, *N*-butan-2-yl-1-(2-chlorophenyl)-*N*-methylisoquinoline-3-carboxamide; PXR, human pregnane X receptor; RIF, rifampicin; RU486, mifepristone; S07662, 1-[(2-methyl-benzofuran-3-yl)methyl]-3-(thiophen-2-ylmethyl)urea; SR12813, {[3,5-*bis*(1,1-dimethyl-ethyl)-4-hydroxyphenyl]-ethenylidene}bisphosphonic acid tetraethyl ester; SW, signal window; TMPP, tri-*p*-methylphenyl phosphate; TPP, triphenyl phosphate.

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http://dx.doi.org/10.1016/j.tiv.2015.07.011
0887-2333/

assays, we took advantage of the reverse transfection methodology (Ziauddin and Sabatini, 2001) in which DNA/carrier complexes are attached to the bottom of cell culture plates and cells take up these complexes after plating (Reinisalo et al., 2006, 2012) (Fig. 1). Here, we optimized the reverse transfection for xenosensor assays with respect to DNA and polyethylene imine 25 (PEI25) carrier amounts, DNA/PEI25 ratios and cell densities. Next, we performed assay validation according to principles by the National Institute of Health (NIH) Chemical Genomics Center (Iversen et al., 2012) and compared the performance of the reverse and conventional transfection. The reverse transfection proved to be an efficient, robust and time-saving method for screening of activators of human xenosensors.

2. Materials and methods

2.1. Chemicals, cell culture materials and plasmids

The synthesis and characterization of triarylphosphates (TMPP, TPP) (Honkakoski et al., 2004) and 1-[(2-methyl-benzofuran-3-yl) methyl]-3-(thiophen-2-ylmethyl)urea (S07662) (Küblbeck et al., 2011b) were described previously. 5-(3,4-Dimethoxybenzyl)-3-phenyl-4,5-dihydroisoxazole (FL81) was prepared and characterized accordingly with 5-benzyl-3-phenyl-4,5-dihydroisoxazole (FL82) (D'Alcontres, 1952; Pulkkinen et al., 2008); FL81: yield 78%, mp = 110.8–112.8 °C, ^{1}H NMR δ 7.64 (m, 2 H), 7.38 (m, 3 H), 6.81 (m, 3 H), 4.97 (m, 1 H), 3.88 (s, 3 H), 3.86 (s, 3 H), 3.32 (dd, 1 H, *J* = 16.6, 10.3), 3.09 (dd, 1 H, *J* = 14.0, 6.2), 3.01 (dd, 1 H, *J* = 16.6, 7.9), 2.85 (dd, *J* = 14.0, 6.8); ^{13}C NMR δ 156.5, 149.0, 147.9 (3 s), 130.0 (d), 129.7, 129.5 (2 s), 128.7, 126.6, 121.4, 112.6, 111.3, 82.0 (6 d), 55.9, 55.9 (2 q), 40.6, 39.3 (2 t). PB was obtained from University Apothecary (Kuopio, Finland). Other compounds were at least of analytical grade: hyperforin (Calbiochem, La Jolla, CA); β-naphthoflavone (BNF), clotrimazole (CLOTR), dexamethasone, methoxychlor, mifepristone (RU486), OME, *o,p′*-DDT, permethrin (PERM), phenytoin (PHN), *N*-butan-2-yl-1-(2-chlorophenyl)-*N*-methyl-isoquinoline-3-carboxamide (PK11195), RIF, {[3,5-bis(1, 1-dimethyl-ethyl)-4-hydroxyphenyl]ethenylidene}bisphosphonic acid tetraethyl ester (SR12813) (Sigma–Aldrich, St. Louis, MO), all steroids (Steraloids, Newport, RI) and simvastatin (Synfine Research, Ontario, Canada). PEI25 and saccharose were from Sigma. Life Technologies/Thermo Fisher Scientific (Waltham, MA) provided Dulbecco's modified Eagle medium (DMEM) cell culture medium and fetal bovine serum (FBS). Delipidated FBS was bought from Hyclone (Logan, UT) and antibiotics and L-glutamine solutions from Euroclone (Pero, Italy). The C3A hepatoma cell line C3A (ATCC CRL-10741) was purchased from LGC Standards (Borås, Sweden). This cell line expresses human AhR endogenously. The CMV promoter-driven expression plasmids for GAL4-LBD fusions of human CAR and PXR, all luciferase reporter genes and the β-galactosidase control reporter have been previously described (Küblbeck et al., 2008, 2011a).

2.2. Conventional transfection and reporter assays

Activation assays for human xenosensors AhR, CAR and PXR were performed in C3A cells cultured on 48-well plates in culture medium (DMEM/1% antibiotics/2 mM L-glutamine/10% FBS). The conventional calcium phosphate-mediated transfection was done essentially as described before (Küblbeck et al., 2008, 2011a). After transfection for four hours, the medium was replaced with fresh DMEM complemented with 1% antibiotics/2 mM L-glutamine/5% delipidated FBS and including either DMSO, positive controls for xenosensors (10 μM OME for AhR; 10 μM FL81 for CAR; 10 μM RIF for PXR) or test chemicals at indicated concentrations (Table 1).

After 24 h of exposure, the cells were disrupted in lysis buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 1% Triton X-100, pH 7.8, supplemented with 1 mM dithiothreitol and phenylmethyl sulfonyl fluoride) and analyzed for luciferase and β-galactosidase activities using the Victor^{2} multiplate reader

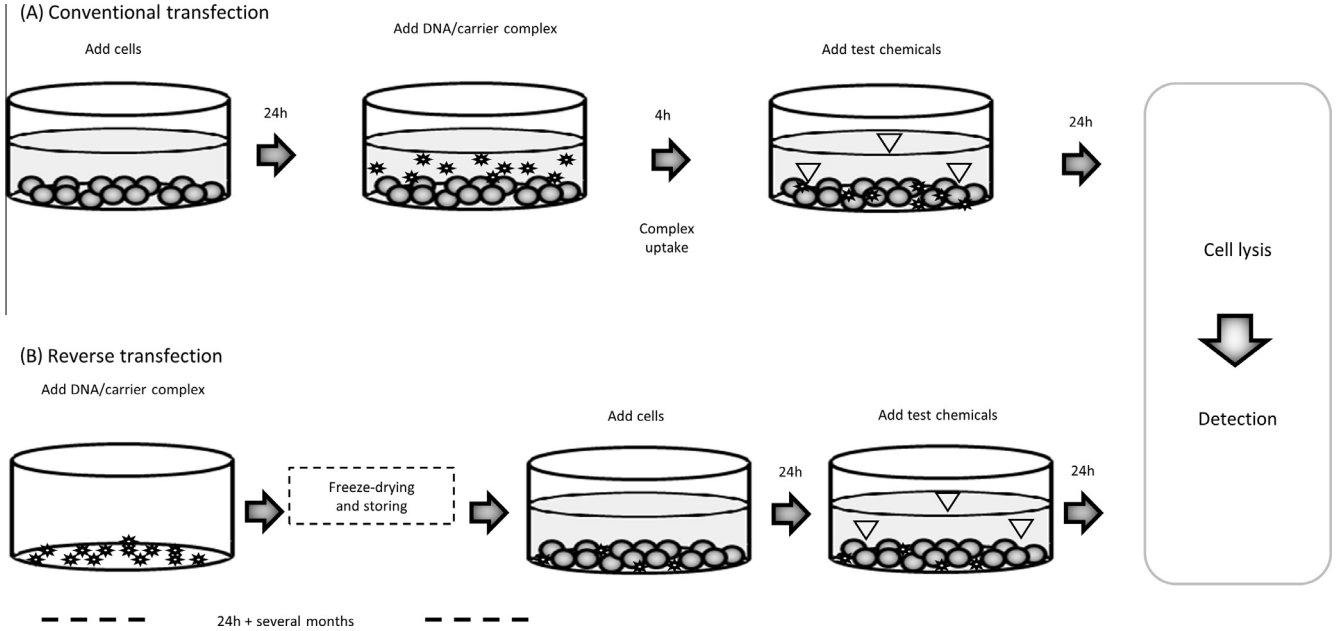

Fig. 1. Comparison between the conventional and reverse transfection procedures. For conventional transfection (A), the cells are seeded and allowed to attach for 24 h. On the next day, DNA/calcium phosphate complexes are prepared and added on top of the cells. After four hours, transfection medium is replaced with fresh medium containing the test chemicals. In reverse transfection (B), the DNA/PEI25 complexes are prepared, added on plates and instantly frozen, freeze-dried overnight and stored for long term at +4 °C until used. The cells are seeded on top of the transfection complexes, which also promote attachment of the cells. The cells are incubated for 24 h after which the medium is replaced with medium containing the test chemicals. In both assays, the cells are exposed to chemicals for 24 h and thereafter assayed for reporter activities.