Antiviral Research 147 (2017) 37–46

Contents lists available at ScienceDirect

Antiviral Research

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

A cell-based high throughput screening assay for the discovery of cGAS-STING pathway agonists

Bowei Liu[a,b], Liudi Tang[c], Xiaohui Zhang[a,d], Julia Ma[a], Mohit Sehgal[a], Junjun Cheng[a], Xuexiang Zhang[a], Yan Zhou[e], Yanming Du[a], John Kulp[a], Ju-Tao Guo[a,**], Jinhong Chang[a,*]

[a] Baruch S. Blumberg Institute, Hepatitis B Foundation, Doylestown, PA, USA
[b] Department of Gastroenterology, Henan Provincial People's Hospital, Zhengzhou University, Zhengzhou, Henan, China
[c] Microbiology and Immunology Graduate Program, Drexel University College of Medicine, Philadelphia, PA, USA
[d] Artificial Liver Center, Beijing You'an Hospital, Capital Medical University, Beijing, China
[e] Biostatistics and Bioinformatics Facility, Fox Chase Cancer Center, Philadelphia, PA, USA

ARTICLE INFO

Keywords:
High throughput assay
STING
Innate immune
Antiviral

ABSTRACT

Stimulator of interferon genes (STING) is an endoplasmic reticulum transmembrane protein that serves as a molecular hub for activation of interferon and inflammatory cytokine response by multiple cellular DNA sensors. Not surprisingly, STING has been demonstrated to play an important role in host defense against microorganisms and pharmacologic activation of STING is considered as an attractive strategy to treat viral diseases and boost antitumor immunity. In light of this we established a HepAD38-derived reporter cell line that expresses firefly luciferase in response to the activation of cyclic GMP-AMP synthase (cGAS)-STING pathway for high throughput screening (HTS) of small molecular human STING agonists. This cell-based reporter assay required only 4 h treatment with a reference STING agonist to induce a robust luciferase signal and was demonstrated to have an excellent performance in HTS format. By screening 16,000 compounds, a dispiro diketopiperzine (DSDP) compound was identified to induce cytokine response in a manner dependent on the expression of functional human STING, but not mouse STING. Moreover, we showed that DSDP induced an interferon-dominant cytokine response in human skin fibroblasts and peripheral blood mononuclear cells, which in turn potently suppressed the replication of yellow fever virus, dengue virus and Zika virus. We have thus established a robust cell-based assay system suitable for rapid discovery and mechanistic analyses of cGAS-STING pathway agonists. Identification of DSDP as a human STING agonist enriches the pipelines of STING-targeting drug development for treatment of viral infections and cancers.

1. Introduction

The genomes of vertebrate animals encode an array of proteins called pattern recognition receptors (PRRs) that recognize pathogen associated molecular patterns upon infection of microorganisms to activate a proinflammatory cytokine response (Akira et al., 2006). This innate cytokine response not only inhibits the proliferation and limits the spread of microorganisms, but also orchestrates the induction of more powerful adaptive immune response to ultimately control the microorganism infections (Chang et al., 2012; Iwasaki and Medzhitov, 2015). Stimulator of interferon genes (STING) is a transmembrane protein localized in the endoplasmic reticulum (ER) membrane and serves as a PRR for cyclic dinucleotides produced by intracellular bacteria or synthesized by the cytoplasmic DNA sensor, cyclic GMP-AMP synthase (cGAS) (Sun et al., 2013; Wu et al., 2013). Binding of the cyclic dinucleotides to STING induces its dimerization and translocation from ER membrane to perinuclear vesicles and subsequently activates NF*k*B and TBK-1/IRF3 (Burdette et al., 2011; Yin et al., 2012). Activation of these signaling pathways induces the expression of type I and type III interferons as well as other proinflammatory cytokines (Tanaka and Chen, 2012). In addition, STING also serves as the adaptor for several other cytoplasmic and nuclear PRRs that recognize DNA to activate innate immune responses (Chen et al., 2016; Kondo et al., 2013). Therefore, STING is a molecular hub for DNA activation of innate immune response and has been demonstrated to play an essential role in host defense against the infection of DNA viruses, retroviruses, intracellular bacteria and protozoa (Cai et al., 2014). Moreover, accumulating evidence suggests that STING also plays an important role in

* Corresponding author.
** Corresponding author.
E-mail addresses: ju-tao.guo@bblumberg.org (J.-T. Guo), jinhong.chang@bblumberg.org (J. Chang).

http://dx.doi.org/10.1016/j.antiviral.2017.10.001
Received 10 August 2017; Received in revised form 28 September 2017; Accepted 1 October 2017
Available online 02 October 2017

host anti-tumor immunity (Corrales et al., 2016).

Recently, several studies showed that activation of STING with small molecular agonists can induce cellular processes that can efficiently inhibit the replication of many DNA and RNA viruses, boost host anti-tumor immune response and enhance the immunogenicity of vaccines (Chang and Guo, 2015; Corrales et al., 2016). These studies prove the concept that pharmacologic activation of STING is an attractive immunotherapeutic approach to treat viral infection and cancer (Corrales and Gajewski, 2015) (Chang and Guo, 2015; Woo et al., 2015). In addition, STING agonists have also been developed as vaccination adjuvants to break immune tolerance against cancer cells and viruses that establish chronic infections in humans (Li et al., 2013).

Currently, there are two classes of STING agonists, cyclic dinucleotides (CDNs) and non-nucleotide small molecules. Bacteria produced cyclic-di-GMP and cyclic-di-AMP are the first STING agonists identified (Burdette et al., 2011). With the discovery of cytosolic DNA sensor cGAS, its catalytic product 2',3'-cGAMP was identified as an even more potent STING agonist (Zhang et al., 2013). Although the various formulations of CDNs have been demonstrated to facilitate the activation of antitumor immune response in mouse models (Fu et al., 2015), their poor cell membrane permeability and metabolic instability limit their biological activity and medical applications. Accordingly, medicinal chemistry efforts have been made to produce novel CDNs that are resistant to the degradation of cellular ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP1) (Li et al., 2014; Lioux et al., 2016). In addition, delivery of CDNs with nanoparticles or liposomes improved their antitumor activities *in vivo* (Hanson et al., 2015). So far, there are only two non-nucleotide small molecular STING agonists, DMXAA and G10. 5,6-dimethylxanthenone-4-acetic acid (DMXAA) was initially discovered and developed as a vascular disrupting agent with antitumor activity in various mouse models, but failed in phase III clinical trials for treatment of lung cancer (Conlon et al., 2013). It was recently identified to be a specific agonist of mouse STING and induced an interferon (IFN)-dominant cytokine response to potently inhibit the replication of influenza A virus, hepatitis B virus and also alphavirus in mice (Cavlar et al., 2013; Conlon et al., 2013; Guo et al., 2015). Interestingly, a genetic study revealed that a single amino acid substitution (S162A) in human STING confers DMXAA sensitivity, which provides a clue for the synthesis of DMXAA analogues as human STING agonists (Gao et al., 2013). G10, or 4-(2-chloro-6-fluorobenzyl)-N-(furan-2-ylmethyl)-3-oxo-3,4-dihydro-2H-benzo[b][1,4]thiazine-6-carboxamide, is a recently identified human STING-specific agonist by high throughput screening. G10 had been demonstrated to induce an antiviral response in human fibroblasts against alphaviruses, but its *in vivo* biological activity and pharmacological property remain to be determined (Sali et al., 2015).

In order to discover small molecular STING agonists with favorable pharmacological properties as the candidates of immunotherapeutics or vaccination adjuvants for viral diseases and cancers, we set out to establish a cell-based cGAS-STING pathway reporter assay and discovered a dispiro diketopiperzine (DSDP) compound that induces proinflammatory cytokine response in a human STING-dependent manner. We have thus demonstrated the robustness and usefulness of this assay as a platform for high throughput screening of the cGAS-STING pathway agonists. In addition, we have also developed a molecular and cellular tool kit for target validation and mechanistic analysis of the identified agonists.

2. Materials and methods

2.1. Cell lines, viruses and reagents

Human hepatoblastoma cell line HepG2 was obtained from ATCC and maintained in Dulbecco's modified minimal essential medium (DMEM)/F12 (Corning) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. HepAD38 is a HepG2-derived stable cell line supporting a tetracycline (tet)-inducible replication of hepatitis B virus (HBV) and was maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum, 400 μg/ml of G418 and 1 μg/ml tetracycline (Ladner et al., 1997). HepAD38/cGAS-STING and HepAD38/cGAS-STINGΔC are HepAD38-derived cell line constitutively expressing human cGAS and STING or a mutant STING with deletion of 39 amino acid residues from carboxyl terminus (STINGΔC) and were maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum, 400 μg/ml of G418, 2 μg/ml of puromycin and 1 μg/ml tetracycline (Guo et al., 2017). Vero (green monkey kidney) cells were maintained in DMEM (Corning) supplemented with 10% fetal bovine serum. THF cells are derived from primary human diploid foreskin fibroblasts (HFF) with extended passage life through expressing of a cDNA encoding the catalytic subunit of human telomerase and were maintained in DMEM with 10% fetal bovine serum (Bresnahan et al., 2000). Human Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient centrifugation (Miltenyi Biotech) from the whole blood of healthy donors (Biological Specialty). Freshly isolated PBMCs were cultured in RPMI-1640 medium (Corning) supplemented with L-Glutamine and 10% fetal bovine serum.

Sendai virus (SenV, strain 52) and human serum derived PRVABC59 strain of Zika virus (ZIKV) were purchased from ATCC. A plasmid containing the yellow fever virus (YFV) 17D complete genomic complementary DNA (cDNA), pACNR/FLYF-17Dx was a gift of Dr. Charles M. Rice at Rockefeller University (Bredenbeek et al., 2003; Rice et al., 1985). A plasmid containing the dengue virus (DENV) serotype 2 New Guinea C strain cDNA (pACYC177-NGC-DENV-2) was a gift of Dr. Pei-Yong Shi at University of Taxis Medical Branch in Galveston (Xie et al., 2013). YFV 17D and DENV-2 virus stocks were produced by electroporation of Huh7.5 cells with *in vitro* transcribed RNAs from the corresponding cDNA constructs, as described previously (Guo et al., 2016).

2′3′-cGAMP, Pam3CSK4, Poly I:C, LPS and Gardiquimod were purchased from Invivogen. G10, a small molecule human STING agonist (Sali et al., 2015), served as a control compound and was purchased from Aobious. Double strand DNA 90 (dsDNA-90) was prepared as previously described (Guo et al., 2017). Dispiro diketopiperzine (DSDP) was purchased from Maybridge. Mouse STING agonist 5,6-dimethylxanthenone-4-acetic acid (DMXAA) was purchased from AdooQ BioScience.

2.2. Establishment of reporter cell lines for high throughput screening

HepAD38/cGAS-STING-derived reporter cell line that expresses firefly luciferase under the control of an ISG54 promoter (HepAD38/cGAS-STING/ISG54Luc) were established by transduction of pGreenFire ISRE lentivector-based transcription reporter system following the manufacturer's protocol (System Biosciences). The pGreenFire ISRE reporter lentiviral vector contains four copies of consensus interferon stimulated response element (ISRE) sequences derived from ISG54 ISRE1, which control the expression of both green fluorescent protein (GFP) and firefly luciferase in response to IRF3 activation and IFN stimulation (Levy et al., 1988). As a negative control, a HepAD38/cGAS-STINGΔC-derived ISG54 promoter reporter cell line (HepAD38/cGAS-STINGΔC/ISG54Luc) was also established using the same pGreenFire ISRE lentivector system.

2.3. High throughput screening assay

HitFinder library containing 16,000 small molecule compounds was screened (Maybridge). HepAD38/cGAS-STING cell line was originally established for investigation of HBV interaction with cGAS-STING DNA sensor pathway (Guo et al., 2017). However, for screening of cGAS and or STING agonists, the HepAD38/cGAS-STING/ISG54 reporter cell line was cultured in complete DMEM/F12 medium containing 1 μg/ml tet throughout the experimental period, which completely prevents HBV