Antiviral Research 144 (2017) 330–339

Contents lists available at ScienceDirect

Antiviral Research

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

Toward the identification of viral cap-methyltransferase inhibitors by fluorescence screening assay

Wahiba Aouadi [a, 1], Cécilia Eydoux [a, 1], Bruno Coutard [a], Baptiste Martin [a], Françoise Debart [b], Jean Jacques Vasseur [b], Jean Marie Contreras [c], Christophe Morice [c], Gilles Quérat [d], Marie-Louise Jung [c], Bruno Canard [a], Jean-Claude Guillemot [a, 1], Etienne Decroly [a, *, 1]

[a] Aix Marseille Université, CNRS, AFMB UMR 7257, Marseille, France
[b] IBMM, CNRS, Université Montpellier, ENSCM, Campus Triolet, Place E. Bataillon, 34095, Montpellier Cedex 05, France
[c] Prestwick Chemical, 67400, Illkirch, Strasbourg, France
[d] UMR "Emergence des Pathologies Virales" (EPV: Aix-Marseille Université - IRD 190 - Inserm 1207 - EHESP), Marseille, France

ARTICLE INFO

Article history:
Received 22 May 2017
Received in revised form
28 June 2017
Accepted 29 June 2017
Available online 1 July 2017

Keywords:
Methyltransferase
Coronavirus
Flavivirus
Inhibitor
HTRF
Antiviral

ABSTRACT

Two highly pathogenic human coronaviruses associated with severe respiratory syndromes emerged since the beginning of the century. The severe acute respiratory syndrome SARS-coronavirus (CoV) spread first in southern China in 2003 with about 8000 infected cases in few months. Then in 2012, the Middle East respiratory syndrome (MERS-CoV) emerged from the Arabian Peninsula giving a still ongoing epidemic associated to a high fatality rate. CoVs are thus considered a major health threat. This is especially true as no vaccine nor specific therapeutic are available against either SARS- or MERS-CoV. Therefore, new drugs need to be identified in order to develop antiviral treatments limiting CoV replication. In this study, we focus on the nsp14 protein, which plays a key role in virus replication as it methylates the RNA cap structure at the N7 position of the guanine. We developed a high-throughput N7-MTase assay based on Homogenous Time Resolved Fluorescence (HTRF®) and screened chemical libraries (2000 compounds) on the SARS-CoV nsp14. 20 compounds inhibiting the SARS-CoV nsp14 were further evaluated by IC_{50} determination and their specificity was assessed toward flavivirus- and human cap N7-MTases. Our results reveal three classes of compounds: 1) molecules inhibiting several MTases as well as the dengue virus polymerase activity unspecifically, 2) pan MTases inhibitors targeting both viral and cellular MTases, and 3) inhibitors targeting one viral MTase more specifically showing however activity against the human cap N7-MTase. These compounds provide a first basis towards the development of more specific inhibitors of viral methyltransferases.

1. Introduction

The severe acute respiratory syndrome coronavirus (SARS-CoV) spread in China in 2003 and was responsible for worldwide outbreak causing over 8000 infected people with a fatality rate around 10% (de Wit et al., 2016). In 2012, a novel human coronavirus named Middle East Respiratory Syndrome Coronavirus (MERS-CoV) emerged in the Arabian Peninsula with secondary infection cases reported in Europe, America, Asia and Africa. MERS-CoV infection resulted in more than 1728 confirmed infected patients till April 2016 with a fatality rate of 36% (de Wit et al., 2016).

CoVs are enveloped viruses possessing a single stranded positive sense RNA genome of approximately 29.7 kb in length (Pan et al., 2008; Van Boheemen et al., 2012). The SARS-CoV initiates its replication cycle after binding to the angiotensin-converting enzyme 2 (ACE2) (Kuhn et al., 2004). After endocytosis, the viral RNA genome is released into the cellular host cytoplasm and translated into two large polyproteins (de Wit et al., 2016; Snijder et al., 2016). The pp1a and pp1ab polyproteins are next cleaved by viral proteases into 11 and 16 non-structural proteins respectively (nsp1 to 16). These proteins form a large replication transcription complex (RTC) which is associated to host cellular

* Corresponding author.
E-mail address: etienne.decroly@afmb.univ-mrs.fr (E. Decroly).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.antiviral.2017.06.021
0166-3542/

proteins (Van Hemert et al., 2008). CoV RTC ensures the replication of the viral genome and the transcription of genomic and subgenomic mRNA. These viral RNAs are polyadenylated at their 3′ end and protected by a 5′ cap structure, which is synthetized by viral enzymes. The CoV capping pathway is thought to follow four sequential steps implicating several nsps: I) the 5′-3′ helicase/NTPase nsp13 hydrolyses first the phosphate γ from the nascent 5'-triphosphorylated RNA (Ivanov and Ziebuhr, 2004); II) a GMP molecule is supposedly transferred to the diphosphorylated RNA by a still unknown guanylyltransferase forming a cap (GpppN) structure; III) the cap is then methylated at N7 position of the guanosine by nsp14 in the presence of methyl donor S-adenosyl-L-methionine (SAM) yielding to a cap-0 (^{7m}GpppN) and S-adenosyl-L-homocysteine (SAH) by-product (Chen et al., 2009); IV) nsp10/nsp16 complex methylates at the ribose 2′OH group of the first transcribed RNA leading to the conversion of the cap-0 (^{7m}GpppN) into the cap-1 (^{7m}GpppN$_{2'om}$) (Bouvet et al., 2010; Chen et al., 2011). *In vitro* assays have deciphered the mechanisms driving the RNA cap methylation in SARS- and MERS-CoV. It follows an obligatory order in which N7-methylation by nsp14 is a pre-requisite for 2′O-methylation by the nsp10/nsp16 complex (Aouadi et al., 2017; Bouvet et al., 2014, 2010).

The guanine N7-MTase activity embedded in the C-terminal domain of SARS-nsp14 has been discovered by yeast trans-complementation assay (Chen et al., 2009). In addition, the N-terminus moiety of nsp14 contains a DEDDh exonuclease (ExoN) domain (Minskaia et al., 2006). The two domains communicate functionally, as truncation experiments showed that the N-terminal region of nsp14 is required for the N7-MTase activity (Chen et al., 2009). Both N7-MTase and ExoN activities have been confirmed by *in vitro* assay showing that the association of nsp10 to nsp14 stimulated >35 fold the ExoN activity while the N7-MTase activity does not depend on the nsp10-nsp14 interaction (Bouvet et al., 2012, 2010; Decroly et al., 2011).

The N7- and 2′O- methylations of the viral mRNA cap are key events for the viral infection. Indeed reverse genetic experiments revealed first that the N7- methylation of cap structures is essential for the synthesis of viral proteins (Case et al., 2016). This observation is corroborated by former biochemical data showing that the N7-methyl guanosine of cap structures is recognized by the eukaryotic translation initiation factor 4E (eIF4E) and participates in the initiation of viral mRNA translation into proteins (Case et al., 2016; Cougot et al., 2004). Accordingly, inhibitors blocking nsp14 N7-MTase activity have been identified by yeast based screening assay on SARS-CoV, and induced a potent antiviral effect demonstrating that nsp14 MTase activity is an attractive antiviral target (Sun et al., 2014). Whereas N7-MTase mutants are replication defective, 2′O-MTase mutants show limited effect on virus replication in cell culture but have an attenuated phenotype in animal models (Li et al., 2013; Menachery et al., 2014; Zhang et al., 2014; Züst et al., 2013). The molecular basis of this attenuated phenotype was recently elucidated: incompletely-capped RNAs have been shown to be recognized by immune sensors such as RIG-I and MDA-5, which trigger innate immunity pathways (Decroly et al., 2012; Schuberth-Wagner et al., 2015; Wu et al., 2013). In turn, RIG-I or MDA-5 induces signalling cascades yielding to the expression of cytokines and type I interferon inducing an antiviral state in neighboring cells. Among the interferon-stimulated gens (ISG), IFIT1 also participates to the restriction of viral replication by sequestrating mis-capped viral RNAs (Pichlmair et al., 2011). Thus cap structure is now considered as a kind of "marker of self" and it is currently admitted that 2′O-MTase inhibitors might help virus clearance by stimulation of the immune response (Decroly et al., 2012; Ferron et al., 2012; Züst et al., 2011).

In this work we first developed an HTRF MTase assay in order to identify compounds inhibiting the N7-MTase activity of SARS-CoV nsp14. Using this system, we screened a library composed of 2000 compounds containing 1280 FDA approved molecules (Prestwick Chemical Library®), 320 natural products and 400 pyridazine-derived compounds. The inhibitory effect of the 20 best compounds was confirmed by a radioactive filter-binding assay, and refined by IC_{50} values determination on SARS nsp14 and human RNA N7-MTase (hRNMT). In addition, the specificity of each compound was further evaluated using the CoV 2′O-MTase (nsp10/nsp16) and the MTases of Dengue and West-Nile flaviviruses as well as the hRNMT involved in the capping of cellular RNAs.

2. Materials and methods

2.1. Description of the libraries

The Prestwick Chemical Library® is a unique collection of 1280 small molecules, mostly approved drugs (FDA, EMA and other agencies) selected for their high chemical and pharmacological diversity as well as for their known bioavailability and safety in humans. The Prestwick Phytochemical Library is a collection of 320 natural products, mostly derived from plants, assembled by medicinal chemists and rich in diverse chemotypes, thus realistic for follow-up chemistry. The Prestwick Pyridazine Library is a collection of 400 innovative pyridazine and pyridazone derivatives, based on a series of carefully selected new original scaffolds. All molecules have been designed to ensure optimal diversity, and are suitable for diverse chemical modification.

2.2. Expression and purification of recombinant proteins

The viral MTases (SARS-CoV nsp14, MERS-CoV nsp14, DENV-3 NS5-MTase, West Nile virus NS5-MTase, DENV-2 NS5-RdRp and human RNA N7-methyltransferase (RNMT) coding sequences were cloned in fusion with a N-terminus hexa-histidine tag in Gateway® plasmids (pDest14 or pDest17, Life technologies). The proteins were expressed in *E. coli* cells and purified following previously described protocols (Aouadi et al., 2017; Bouvet et al., 2010; Ma et al., 2015; Milhas et al., 2016; Peyrane et al., 2007; Selisko et al., 2006). MERS-CoV nsp14 was produced and purified as follows: the protein were expressed in Arctic Express *E. coli* strain (Agilent) at 13 °C during 24 h after addition of 50 μM IPTG. The bacteria were pelleted (13,000× g, 4 °C, 5 min), and lysed by sonication in appropriate buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5% glycerol, 20 mM imidazole, 5 mM β-mercaptoethanol, 1 mM PMSF, 10 μg/ml DNase-I and 0.25 mg/ml lysozyme). After clarification (50,000× g, 30 min at 4 °C) the recombinant protein was purified by immobilized metal affinity chromatography (IMAC) on 1 ml of HisPurTM Cobalt Resin (Thermo Scientific) followed by size exclusion chromatography on a Superdex 200 (GE healthcare) equilibrated in (30 mM HEPES pH (7.5), 300 mM NaCl and 5% glycerol). The purified proteins were analyzed on SDS-PAGE gels after Coomassie blue staining (Fig. 1 and Fig. S1).

2.3. HTRF screen on SARS CoV nsp14 MTase

For the initial screening, 400 nl of each compound resuspended in 100% DMSO at 1 mM were dispensed in the reaction volume (5% final DMSO, 50 μM compound) using a Mosquito Crystal pipetting robot platform (TTP labtech). DMSO, sinefungin (20 μM) and SAH (2 μM) were used as controls.

The enzymatic reaction was performed in 8 μl. 3 μl of mix (Buffer + SARS nsp14 at 5 nM final concentration) were added in the assay wells, containing previously dispensed inhibitors (0.4 μl), using a Biomek NX MC pipetting robot (Beckman). The reaction