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Screening and characterization of molecules that modulate the biological activity of IFNs-I

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ABSTRACT

Type I Interferons (IFNs-I) are species-specific glycoproteins which play an important role as primary defence against viral infections and that can also modulate the adaptive immune system. In some autoimmune diseases, interferons (IFNs) are over-produced. IFNs are widely used as biopharmaceuticals for a variety of cancer indications, chronic viral diseases, and for their immuno-modulatory action in patients with multiple sclerosis; therefore, increasing their therapeutic efficiency and decreasing their side effects is of high clinical value. In this sense, it is interesting to find molecules that can modulate the activity of IFNs. In order to achieve that, it was necessary to establish a simple, fast and robust assay to analyze numerous compounds simultaneously. We developed four reporter gene assays (RGAs) to identify IFN activity modulator compounds by using WISH-Mx2/EGFP, HeLa-Mx2/EGFP, A549-Mx2/EGFP, and HEp2-Mx2/EGFP reporter cell lines (RCLs). All of them present a Z′ factor higher than 0.7. By using these RGAs, natural and synthetic compounds were analyzed simultaneously. A total of 442 compounds were studied by the Low Throughput Screening (LTS) assay using the four RCLs to discriminate between their inhibitory or enhancing effects on IFN activity. Some of them were characterized and 15 leads were identified. Finally, one promising candidate with enhancing effect on IFN-α/-β activity and five compounds with inhibitory effect were described.

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1. Introduction

Interferons are species-specific glycoprotein family that play an important role against viral infections as a first line defence system (Billiau, 2006). They protect the organism from diverse pathogens, and also participate in the adaptive immune response and act in both an autocrine and paracrine fashion alerting the surrounding cells to the presence of pathogens. IFNs bind to their specific receptors on the cell surface and initiate a signaling pathway which concludes with the activation of more than 300 interferon-stimulated genes (ISG) in the target cells (Lopez and Hermesh, 2011; Richards and Macdonald, 2011) to develop the anti-viral, anti-proliferative and immune-modulator responses. Due to their properties, these cytokines are used as biopharmaceuticals to treat

Abbreviations: APA, antiproliferative assay; AVA, antiviral assay; C+, positive control; C-, negative control; CC, compound control; CWT, cells without treatment; DMSO, dimethyl sulfoxide; EGFP, enhanced green fluorescent protein; EnE, enhancer effect; FCS, fetal calf serum; FSC, foward scatter; GepA, Gephryronic acid A; hIFNs-I, human type I interferons; HTS, high throughput screening; IFNs, interferons; IFNs-I, type I interferons; IFN + C, co-incubation control; IRF7, interferon response factor 7; ISG, interferon-stimulated genes; ISRE, IFN-stimulated response elements; LC–MS, liquid chromatography–mass spectrometry; LTS, low throughput screening; mCHR, minimal concentration of compound giving the highest modulator response of IFN activity; NSE, not significant effect; PBS, phosphate-buffered saline; Pella, Pellasorem; PI, propidium iodide; RCLs, reporter cell lines; RGAs, reporter gene assays; rhIFN, recombinant human interferons; RsE, residual effect; RvE, reversal effect; SCC, side scatter; Sula, Sulasorem; TubI, Tubulysin I; VioB, Vioprolide B; VSV, vesicular stomatitis virus; σ_{pos}, standard deviation for positive control; σ_{neg}, standard deviation for negative control; μ_{pos}, mean signal for positive control; μ_{neg}, mean signal for negative control.

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viral and tumor pathologies. In order to fulfill their role as biotherapeutics, the final product should undergo quality controls to ensure its biological activity, and correspondingly assure the correct relation between IFN dose and therapeutic efficacy (Billiau, 2006; Larocque et al., 2011).

Currently, IFN potency is determined through the antiviral assay (AVA), as recommended by the European Pharmacopeia (2009). However, AVAs are subject to high intra- and inter-test variations and require virus manipulation under biosafety level 2 conditions. Besides, AVAs specifically reflect an IFN's ability to protect cells from virus attacks, a property that must not be related to antitumor activity and immune-modulation. With the purpose of replacing this assay, we previously developed four human RCLs to measure IFN potency, where the enhanced Green Fluorescent Protein (EGFP) is driven by the Mx2 promoter. The Mx genes are known to react consistently to type I-IFN in a variety of cells and are used as hallmarks for ISG activation (Asano et al., 2003; Pulverer et al., 2010). WISH-Mx2/EGFP, HeLa-Mx2/EGFP, A549-Mx2/EGFP, and HEp2-Mx2/EGFP RCLs express the specific receptor for IFN on their cell surface. After IFN incubation, the Mx2 promoter is activated and consequently EGFP is expressed. Hence, the percentage of EGFP-expressing cells, quantified by flow cytometry, is directly correlated with IFN potency (Bürgi et al., 2011; Bürgi et al., 2012; Kugel et al., 2011).

However, there are some disadvantages to the use of IFNs as biopharmaceuticals. These molecules have a rapid clearance, so in order for them to achieve their therapeutic effect it is necessary to use high doses repeatedly. Furthermore, significant side effects were registered as a consequence of these dosing applications, often causing the interruption of the treatment without reaching the therapeutic objective.

IFN-αs, as members of the type I IFN family, are excessively produced in some autoimmune diseases (Banchereau and Pascual, 2006; Meyer, 2009) and this overproduction, contributes to the pathogenesis and symptoms of the disease. Thus, while human type I interferons (hIFNs-I) have beneficial clinical effects, their side effects reduce their use as biopharmaceuticals. Therefore, increasing their therapeutic efficacy and decreasing their side effects would be of significant clinical value. In this sense, it is of interest to find molecules to modulate IFN activity. In order to create a simple, fast and robust assay to analyze many compounds simultaneously, four RCL have been developed to be used in a LTS or high throughput screening (HTS) assay format (Szymański et al., 2012; Martis et al., 2011; Mishra et al., 2008). HTS typically refers to a process in which a large number of chemicals are tested with high efficiency to identify biologically active small molecules as candidates for further validation in additional biological or pharmacological experiments (An and Tolliday, 2010). The four cell lines were engineered to express EGFP under the Mx2 promoter regulation and LTS assays were established. Assays for LTS require adequate sensitivity, reproducibility, and accuracy to discriminate among a large number of compounds that include the entire range of IFN activity. The Z′ factor was calculated as a characteristic parameter to define the performance of the assay (Zhang et al., 1999). Libraries composed of natural and synthetic compounds were screened through LTS assays. Fifteen hit compounds were identified and their specific properties were characterized. Cytotoxicity, mCHRs, antiviral and antiproliferative activities, residual and reversal effects, as well as their influence on cell cycle were studied.

2. Materials and methods

2.1. Cell lines

WISH-Mx2/EGFP, HeLa-Mx2/EGFP, A549-Mx2/EGFP and HEp2-Mx2/EGFP RCLs were previously described (Bürgi et al., 2011; Bürgi et al., 2012). Specifically, the best clone from each RCL was employed: WISH-Mx2/EGFP (L1G3), HeLa-Mx2/EGFP (C6C3), A549-Mx2/EGFP (L2G9), HEp2-Mx2/EGFP (L1G5). Cell clones were grown and maintained in Minimal Essential Medium (MEM) −WISH-Mx2/EGFP and HEp2-Mx2/EGFP- or Dulbeccoís Modified Eagle Medium (DMEM) −HeLa-Mx2/EGFP and A549-Mx2/EGFP- supplemented with 10% (v/v) fetal calf serum (FCS) and 2 mM glutamine.

2.2. Interferons

Recombinant human IFN-α2a (rhIFN-α2a) was obtained from Zelltek S.A. (Santa Fe, Argentina), and rhIFN-β1a (Avonex) was purchased from Biogen (USA).

2.3. Libraries of compounds

Natural compounds were obtained from the Department of Chemical Biology (CBIO), Helmholtz Centre for Infection Research, Germany. The collection of natural products used for screening consists of 154 compounds that had been isolated at the Helmholtz Centre for Infection Research from cultures of myxobacteria during the past 30 years (Reichenbach and Höfle, 1999).

Compounds were checked for integrity and purity (> 95%) by LC–MS and were seeded into a 96-well plate at a concentration of 0.5 mg/ml in DMSO. Among the 154 natural compounds studied, 5 of them proved to be highly effective in cell culture systems. They are VioprolideB (VioB), Tubulysin I (TubI), Sulasoren (Sula), Gephyronic acid A (GepA) and Pellasoren (Pella). Structures are ilustrated in Fig. 1, with the exception of Sula, since its structure has not been published yet.

Synthetic compounds were obtained from the Institute of Organic Chemistry, Clausthal University of Technology, Germany. A total of 288 compounds were assayed. Compounds were available into a 96-well format at a concentration of 18 mM in DMSO. Ten of the 288 synthetic compounds studied were found to be highly effective in our cell culture systems. These were P5D7, P5H10, P6C11, P6H1, P28E1, P28E9, P28F7, P28G6, P28H3 and P28H7 and their structures are depicted in Fig. 1. Their synthesis and characterization were previously described (Nechai et al., 1997; Potkin et al., 1991; Zapol'skii et al., 2012, 2004, 2015).

2.4. Validation of cell line-based RGAs

The most widely accepted measurement of an assaysíquality and readiness is the Z′ factor (Zhang et al., 1999; Entzeroth et al., 2009; An and Tolliday, 2010). The Z′ factor is an indicator of the quality of any given assay, and it measures the separation of a positive signal of the sample and the background control in the absence of a test compound. The Z′ factor was estimated according to Eq. (1):

$$Z' = 1 - [(3\sigma_{pos} + 3\sigma_{neg})/(|\mu_{pos} - \mu_{neg}|)](1) \quad (1)$$

where μ_{pos} is the mean signal for the positive control, μ_{neg} is the mean signal for the negative control, σ_{pos} is the standard deviation for the positive control, and σ_{neg} is the standard deviation for the negative control. Negative controls were performed by adding MEM or DMEM culture medium supplemented with 2% (v/v) FCS whichever was applicable. Positive controls were performed by incubating cells with rhIFN-α2a or rhIFN-β1a at a specific concentration which produced 50% of EGFP response for each RGA: 40 IU/ml and 12 IU/ml for WISH-Mx2/EGFP, 2.5 IU/ml (of both IFNs) for A549-Mx2/EGFP; 2.5 and 1.5 IU/ml for HeLa-Mx2/EGFP and, 250 and 50 IU/ml for HEp2-Mx2/EGFP, respectively. $Z' \geq 0.5$ indicates an excellent assay while an assay with $0 < Z' < 0.5$ is considered marginal and may be suitable for further screening but

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