Journal of Biotechnology 233 (2016) 6–16

Contents lists available at ScienceDirect

Journal of Biotechnology

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

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

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ARTICLE INFO

Article history:
Received 5 February 2016
Received in revised form 9 June 2016
Accepted 21 June 2016
Available online 23 June 2016

Keywords:
Cell-based RGA
hIFN-I
Natural and synthetic compounds
Low Throughput Screening
Mx2/EGFP

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.

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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http://dx.doi.org/10.1016/j.jbiotec.2016.06.021
0168-1656/

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):

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

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

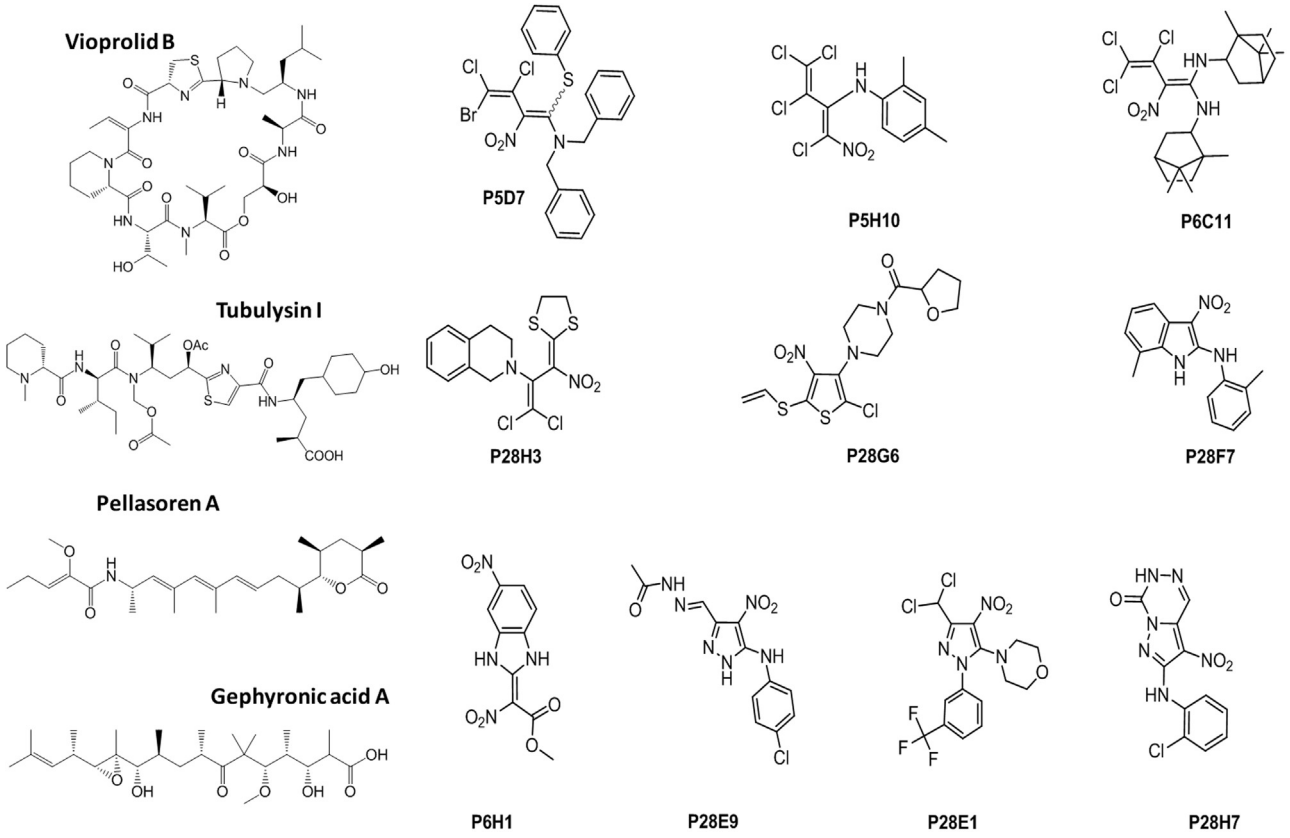


Fig. 1. Chemical structures of the most active natural and synthetic compounds.
The natural compounds (Vio B, Tub I, Pella A, Gep A) are shown on the left and the synthetic compounds (P5D7, P5H10, P6C11, P28H3, P28G6, P28F7, P6H1, P28E9, P28E1, P28H7) on the right.

requires optimization. Assays with $Z' < 0$ are not suitable for screening (Entzeroth et al., 2009; An and Tolliday 2010).

2.5. Cell-based LTS from libraries of compounds

Two libraries composed of 154 natural compounds and 288 synthetic compounds each were analyzed employing the four previously developed RGAs (Bürgi et al., 2011; Bürgi et al., 2012). Assays on 96-well plates were automated. Cells were seeded into 96-well plates at 2.5 x 10^4 cell/well in 0.1 ml of the appropriate medium and incubated at 37 °C and 5% CO_2 for 24 h. Supernatants were removed and rhIFN-α2a or rhIFN-β1a were added, as appropriate, at a potency which produces 50% of the EGFP response. For A549-Mx2/EGFP cells, 2.5 IU/ml were used for both rhIFNs, while 2.5 and 1.5 IU/ml were used for HeLa-Mx2/EGFP cells, for WISH-MX2/EGFP cells 40 and 12 IU/ml, and for HEp2-Mx2/EGFP cells 250 and 50 IU/ml of rhIFN-α2a and rhIFN-β1a were added, respectively. Immediately after IFN addition, compounds were added at 8 μM final concentration using a liquid handling robot Biomek® FXP Laboratory Automation Workstation (Beckman Coulter, USA). Plates were incubated at 37 °C, 5% CO_2 during 24 h. Supernatants were discarded, and cells were trypsinized and properly suspended in 0.2 ml PBS to homogeneity. The percentage of EGFP positive cells was measured using a BD™ LSR II flow cytometer (Beckton Dickinson, USA) with BD™ HTS option (Beckton Dickinson, USA) coupled for high throughput sample acquisition. BD FACSDiva™ software was used for data acquisition and FlowJo version 7.6.5 software was used for data analyses. For each sample 1,000 events were collected gating on the FSC vs SCC dot plot, and a fluorescence histogram plot was used to estimate the percentage of EGFP. On each plate, 4 negative and 4 positive controls were included and they were located in column 12 considering the robot provision system. Negative controls were performed by treating cells with assay medium and positive controls were performed as described above by incubating cells with the rhIFN concentration that provides a signal of 50% of EGFP response. Additionally, the sensitivity to DMSO (the solvent used to preserve the compounds in the libraries) was previously determined for each RGA. Taking this into account, the final concentration of DMSO used during the screening was significantly lower than the toxic levels determined (supplementary material).To minimize errors (false positive or negative hits) two independent screenings were performed for each combination of the RCL and the corresponding rhIFNs (rhIFN-α2a or rhIFN-β1a).

2.6. Compound toxicity assay using crystal violet dye

The toxicity of each compound was determined using crystal violet staining. This method is useful for the rapid detection of highly toxic compounds at 24 h. Since the dye stains viable cells, the less intensively colored cells indicate compound toxicity. Cells were seeded at 2.5 x 10^4 cell/well in 0.1 ml growing medium and incubated at 37 °C and 5% CO_2 during 24 h. Supernatants were discarded and two-fold serial dilutions of each compound were evaluated in triplicate. The range of concentrations of the compounds were the following VioB 930 – 14.5 μM, TubI 100 – 1.6 μM, GepA 883 – 13.8 μM, Pella and Sula 1,000 – 15.6 μM and all the synthetic compounds 14 – 0.2 μM. Cells were incubated for 24 h at 37 °C and 5% CO_2. The supernatants from each well were discarded and 50 μl/well of crystal violet dye was added and incubated at 37 °C and 5% CO_2 for 30 min. The dye was removed and plates were washed generously with water. The plates were left to dry and the color intensity was measured using a digital camera. Untreated cells

were considered as negative control. The non-toxic limit concentration was calculated as the highest concentration of compound which produced the same color intensity than that of the negative control. These concentrations were then used for further characterization of compounds.

2.7. mCHR

The minimal concentration of each compound that provides the highest response to modulate IFN activity is defined as the lowest concentration that provides the highest modulation of EGFP read-out. This concentration was evaluated by RGA following the protocol described above. Two-fold serial dilutions from each compound were tested in triplicate in the presence of the described rhIFN potency. The following controls were also included in triplicate: positive control (C+, IFN-treated cells), negative control (C-, untreated cells) and compound control (CC, cells treated only with the corresponding compound at its mCHR). The EGFP percentage from each sample was measured according to the procedure described above.

2.8. Analysis of temporal action using the WISH cell-derived RGA

An assay was used to analyze if a compound's effect persists even after the compound is removed from the system. Therefore, IFN was added at different times after compound incubation and removal, and the residual effect of the compounds was characterized. To accomplish the temporal action of compounds, WISH-Mx2/EGFP reporter cells were seeded in 96-well plates (2.5×10^4 cells/well) and incubated during 24 h at 37 °C with 5% CO_2. Supernatants were discarded and the compounds were added at their previously defined mCHR (prepared in MEM medium supplemented with 2% (v/v) FCS). After 1 h of incubation, compounds were removed, and each well was washed twice with culture medium. rhIFN-β1a (12 IU/ml) was added at 0, 1, 2 or 3 h after washing. IFN-treated cells were further incubated for 24 h. Finally, cells were detached by using trypsine and correctly resuspended in 0.2 ml of PBS. Then, EGFP expression was determined. In this assay, controls were also included in triplicate as follows: IFN + C: co-incubation control (cells treated with IFN plus compound during the same time); C+: positive control (IFN-treated cells); C−: negative control (cells without treatment) and CC: compound control (cells treated with the corresponding compound at its mCHR). For statistic validation the ANOVA test followed by Tukey's multiple comparison test was employed.

2.9. Analysis of compound effect after IFN activation using the WISH cell-derived RGA

IFNs bind their specific receptor to start the activation pathway through which they develop their biological activity. That pathway concludes in the cell nucleus with the induction of a set of immediate-early response genes.

After triggering the IFN pathway, the effect of different compounds was evaluated by adding them at different times post cytokine incubation using WISH cell based-RGA. For that, WISH-Mx2/EGFP reporter cells were seeded in 96-well plates (2.5×10^4 cells/well) and incubated during 24 h at 37 °C with 5% CO_2. Supernatants were discarded and rhIFN-β1a was added, at a concentration of 12 IU/ml in MEM medium supplemented with 2% FCS. rhIFN-β1a was incubated during 1, 2 or 3 h in different wells at 37 °C with 5% CO_2. After IFN incubations, compounds were added at their mCHRs without removing the rhIFN-β1a. Cells were incubated during 24 h at 37 °C with 5% CO_2. Cells were trypsinized, carefully suspended in 0.2 ml PBS and then EGFP expression was measured. In addition, the following controls were included: IFN + C: co-incubation control (cells treated with IFN plus compound at the same time); C+: positive control (IFN-treated cells); C-: negative control (cells without treatment) and CC: compound control (cells treated with the corresponding compound at its mCHR). For statistic validation the ANOVA test followed by Tukey's multiple comparison test was employed.

2.10. Antiviral activity assay (AVA)

The antiviral activity of IFNs-I in the presence of different compounds was estimated by the protective effect on WISH cells infected with vesicular stomatitis virus (VSV) following the recommendation of the European Pharmacopeia (2009) and compared to antiviral activity of IFN standards without the addition of compounds. WISH cells were seeded at 2.5×10^4 cells/well in 96-well plates and incubated 24 h at 37 °C with 5% CO_2. After removing supernatants, references and samples were added. As references, rhIFN-β1a and rhIFN-α2a were used in a range from 5 to 0.16 IU/ml and from 25 to 0.78 IU/ml, respectively, performing six two-fold serial dilutions. Samples were prepared employing the respective concentrations of rhIFN used in the standard curve plus the compound at a concentration corresponding to its mCHR. After 6 h of incubation, VSV was added. Virus replication was allowed to proceed until the cytopathic effect was clearly observable in control wells with no IFN added (cytopathic effect control). An incubation time of 18–20 h at 37 °C with 5% CO_2 was needed to reach this effect. The medium was discarded and cells were fixed and stained simultaneously with a solution of 0.75% (w/v) crystal violet in 40% (v/v) methanol. After 10 min, plates were washed with water and the remaining dye was solubilized in 20% (v/v) acetic acid. Plates were read at 540 nm with a microtiter plate reader and the signal intensity of each dilution was reported as the mean of the absorbance measured in triplicate. Controls were carried out assaying cells without IFN (cytopathic effect control) or without IFN and virus (cellular control).

2.11. Antiproliferative activity assay (APA)

WISH-Mx2/EGFP cells were seeded (6.25×10^2 cells/well) and simultaneously incubated with rhIFN-β1a or rhIFN-α2a plus the selected compounds in MEM medium supplemented with 10% (v/v) FCS during 96 h at 37 °C, 5% CO_2. IFN standard curves were performed using six two-fold serial dilutions, from 1,200 to 37.5 IU/ml for rhIFN-β1a and from 12,000 to 375 IU/ml for rhIFN-α2a. Each compound was evaluated at its mCHR defined in the previous assays. Cell proliferation was determined using a Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, USA). The colorimetric reaction proceeded during 3 h at 37 °C, 5% CO_2 and the plate was read at 492 nm with a microplate reader using 600 nm as reference absorbance. The assay was reproduced in triplicate. The following controls were included: proliferation control (untreated cells) and compound control (cells treated only with compound).

2.12. Analysis of compound effect using a validated murine RCL (NIH3T3 IRF7-mCherry)

In order to analyze the selectivity and specificity of the new RCL to detect compounds that function as inhibitors or enhancers of the IFN-I activity, compounds were studied using a murine RCL (NIH3T3 IRF7-mCherry). The assay was carried out essentially following the procedure summarized by Rand et al. (2012). Thus, cells were seeded (5×10^5 cell/well) in 12-well plates in DMEM medium supplemented with 10% (v/v) FCS and incubated during 24 h at 37 °C, 5% CO_2. Supernatants were removed and samples were added in DMEM medium supplemented with 2% (v/v) FCS. Each compound was evaluated using its mCHR and incubated in the presence of

mIFN (1,000 U/ml). Some pictures were captured using a confocal fluorescence microscope at different time's "post-incubation" with mIFN and the compound (40, 160 and 1360 min). The following controls were assayed: negative control (cells without treatment), positive controls (cells only treated with mIFN) and compound control (cells only treated with the corresponding compound at its mCHR).

2.13. Cell cycle analysis

In addition to the AVA and APA, cell cycle analysis was performed only with WISH-Mx2/EGFP cells. The cells were seeded in 12-well plates (5×10^5 cell/well) in MEM medium supplemented with 10% (v/v) FCS and incubated during 24 h at 37 °C, 5% CO_2. Supernatants were removed and samples were added in MEM medium supplemented with 2% (v/v) FCS and incubated during 24 h at 37 °C, 5% CO_2. Each compound was evaluated using its mCHR and incubated in the presence of rhIFN-α2a (60 IU/ml) or rhIFN-β1a (20 IU/ml). The following controls were included: cells incubated with each compound (CC), cells without any treatment (CWT), and cells treated only with each cytokine (rhIFN-α2a and rhIFN-β1a). Samples and controls were run in triplicate. For cell cycle distribution, cells were fixed by dripping in 70% (v/v) ethanol and incubating for 30 min at 4 °C. Fixed cells were centrifuged, counted and diluted to 1×10^6 cell/ml final cell density in PBS-EDTA 2 mM. After that, cells were filtered using a 0.45 μm filters to remove cell aggregates. Propidium iodide (PI) was used as an intercalating dye. Cells were stained with a solution containing 50 μg/ml RNAseA (Sigma-Aldrich, USA) and 50 μg/ml PI (Invitrogen, USA) in PBS for at least 1 h in the dark at 37 °C. The DNA content was analyzed by flow cytometry employing a CyAn™ ADP Analyzer (Beckman Coulter, USA) equipped with 488 nm emitting laser. The acquisition rate was adjusted to 80–120 events per second. Summit v3.4. software was used for data collection. For each sample at least 20,000 total cells were recorded. Doublets and cellular debris were discarded from analyses. Cell cycle phase distribution (G1, G2-M and S) was obtained employing Flowjo™ v7.6.5 software with the cell cycle analyses package. Data were exported and statistic treatment were applied by using Prism 5.0 software (GraphPad software) between cell cycle phases obtained from compound plus IFN-treated cells and the control situation (cells treated only with IFN).

2.14. Statistical analysis

All experiments were performed in triplicates and the mean values with standard errors were calculated. ANOVA test followed by the corresponding post-hoc test were applied for all data using Graph Pad Prism version 5.0. The proper post-hoc test is indicated in each section where it was used.

3. Results and discussion

3.1. Validation of cell line-based RGAs

For cell based assays previously optimized RGAs were used. WISH-Mx2/EGFP was the first RCL developed to measure rhIFNs-I biological activity. This system requires the use of a standardized cell line (WISH cells) – suggested by Pharmacopoeia (European Pharmacopeia, 2009) to be used for human IFN-β potency analysis- and the simultaneous use of the sensitive reporter gene eGFP. Since several advantages were demonstrated by this RGA when compared to the antiviral activity assay and other reporter systems (Bürgi et al., 2012), a set of new human reporter cell lines derived from different tissues were developed. These RGAs were designed aiming to evaluate how IFNs induce their actions throughout the human body and, in addition, to analyze and identify several compounds that potentially modulate IFN activity. Therefore, the following cell lines were also used to develop new RGAs (Bürgi et al., 2011): A549 (lung cancer cells), HEp-2 (epidermoid larynx carcinoma cells) and HeLa (cervical adenocarcinoma cells).

Table 1
Z′ factor calculated for each RGA.

RGA	Z′ Factor	
	rhIFN-α2a	rhIFN-β1a
WISH-MX2/EGFP (L1G3)	0.87 ± 0.08	0.85 ± 0.08
HeLa-Mx2/EGFP (C6C3)	0.85 ± 0.07	0.82 ± 0.09
A549-Mx2/EGFP (L2G9)	0.87 ± 0.07	0.86 ± 0.06
HEp2-Mx2/EGFP (L1G5)	0.73 ± 0.11	0.86 ± 0.11

Z′ factor was estimated for each RCL in triplicate as:
$Z' = 1 - [(3\sigma_{pos} + 3\sigma_{neg})/(|\mu_{pos} - \mu_{neg}|)]$ 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 positive control, and σ_{neg} is the standard deviation for the negative control.

In an attempt to validate the RCLs in LTS approaches, several replicates of positive and negative controls were run. The negative control represents the background of the assay whilst the positive control reflects the IFNs-induced response. Controls were assayed in quadruplicates and four independent experiments were carried out in order to assess the reproducibility parameter and the signal variation at the two extremes of the activity range. The data obtained from these controls was used to determine the limits of the assay, aiming to detect compounds that exert a positive or negative effect on the cytokine response.

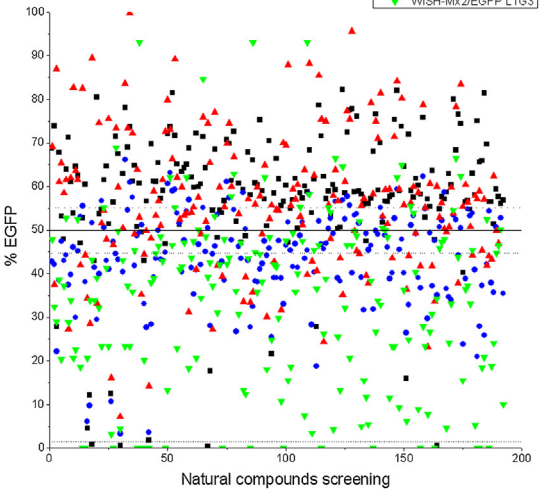
IFNs-derived RGAs showed Z′ values higher than 0.7 (Table 1). A general consideration establishes that the Z′ factor should be higher than 0.5 in order to accept an assay as appropriate to employ in LTS format. Our data demonstrates the validity for LTS analysis and reflect the high degree of readiness and aptitude of the assay to identify active compounds. This also ensures that the assay format has been properly implemented and that the assay shows sufficient dynamic range and acceptable signal variability to provide useful data (Zhang et al., 1999).

3.2. Cell-based LTS from libraries of compounds using the validated RGAs

In general, the purpose of screening compounds is to search for "hits" or primary active compounds that exhibit non-promiscuous behavior and exceed a threshold value in a given assay. Each compound was tested in order to determine its effects on IFN response using the four cell line-derived RGAs. The EGFP fluorescence values obtained from each one were compared to a cut-off value or threshold (Brideau et al., 2003). Upper and lower threshold values were calculated as means of the positive control plus or minus three times their standard deviation with a confidence limit of 99.73%. Fig. 2A, shows an example of the screening of natural compounds using the 4 RCL by activating the system with rhIFN-β1a. Hits were identified by calculating threshold values of positive controls and applying hit selection criteria; e.g., a compound was registered as a hit when it showed a compound signal $>\mu_{pos} + 3\sigma_{pos}$ or $<\mu_{pos} - 3\sigma_{pos}$, where μ_{pos} and σ_{pos} stand for the mean of positive controls and the corresponding standard deviation, respectively. Since replicates reduce the number of false negatives without increasing the number of false positives, compounds were re-evaluated in a second screening. A compound was confirmed as a hit if both replicates showed the previously defined criteria.

Taking the previous information into account, a subset of 442 compounds was analyzed (from libraries of natural and synthetic origin). The natural library was completely analyzed because its size admitted a thorough study (154 compounds). For the synthetic

A



B

RCL	Library					
	Natural			Synthetic		
	rhIFN-α2a	rhIFN-β1a	rhIFN-α2a rhIFN-β1a*	rhIFN-α2a	rhIFN-β1a	rhIFN-α2a rhIFN-β1a*
WISH-Mx2/EGFP (L1G3)	54	31	25	46	50	40
HeLa-Mx2/EGFP (C6C3)	35	28	24	25	32	22
A549-Mx2/EGFP (L2G9)	51	40	18	29	33	22
Hep2-Mx2/EGFP (L1G5)	48	24	19	45	51	30
All RCL	32	21	14	23	32	14

Fig. 2. Library screening using different RGAs induced by rhIFN.
A: The library of natural compound was screened through the 4 RGAs and induced with rhIFN β1a at potency that produce 50% EGFP response. The continuous black line represents the mean signal of positive controls, the dot lines represent the upper and lower threshold values (mean signal of positive controls plus or minus standard deviation, respectively) and the dash lines represent the mean signal of the negative controls.
B: Percentage of responding compounds (hits) from the natural or the synthetic libraries that were analyzed by RCL after their induction with rhIFN-α2a or rhIFN-β1a. (*) Represents the percentages of hits that showed the same behavior when rhIFN-α2a or rhIFN-β1b were individually used to induce each RCLs or all of them.

library, with 10,000 compounds, a pre-screening was carried out using a HeLa-MxLuciferase RGA performed by the owner of the library. This pre-screening allowed the selection of a lower number of compounds to be tested by the RGA developed. To determine their effects, data was compared to a cut-off value defined as previously explained. All the RGAs allowed the successful identification of compounds showing a positive or a negative effect to modulate IFN activity. Compounds that exceeded the upper threshold were considered potential enhancers of IFN activity. They showed EGFP values higher than that of the positive control in accordance to a higher activation of the specific Mx2 promoter. On the contrary, compounds exhibiting EGFP values lower than that of the inferior cut-off were designated as putative inhibitors of IFN activity. These compounds showed less activation of EGFP expression. Fig. 2B summarizes the percentage of hit rate from each library, which was calculated for every reporter cell line in the presence of rhIFN-α2a or rhIFN-β1a. In addition, for both types of IFNs, the WISH cell-based RGA identified the highest number of responding compounds: 54 from the natural library and 115 from the synthetic library. HeLa, Hep2 and A549 cell-based RGA identified 37, 29 and 28 compounds from the natural library and 63, 86 and 63 from the synthetic library, respectively (data not shown). Differences between RGA performances are probably due to particular characteristics of each cell line and they may be related to their susceptibility to the compounds. Furthermore, 21 compounds from the natural library and 40 compounds from the synthetic library showed to equally modulate both rhIFN activities through the four RGAs assayed.

Furthermore, it is important to highlight that the extremely high hit rates achieved with our RGAs could be explained by the fact that the compounds herein screened are a subset from larger libraries that were previously screened by another reporter systems (Bollati-Fogolín and Müller, 2005; Seo et al., 2009). In addition, the information about the families of compounds provided by the library's holders was taken into account for the selection of the subset of compounds that were further screened (data not shown).

Considering those compounds that showed reproducible higher responses along the screening by employing the four RCL and both rhIFNs, five natural and ten synthetic compounds were selected and further characterized for biological properties regarding their effect on IFNs potency. Fourteen from those fifteen selected compounds were inhibitors (VioB, Sula, GepA and Pella from the natural library and P5D7, P5H10, P6C11, P6H1, P28E1, P28E9, P28F7, P28G6, P28H3 and P28H7 from the synthetic library); the remaining one was an enhancer (TubI from the natural library). TubI has been reported to be a strong inhibitor of tubulin polymerization (Khalil et al., 2006) and GepA was described as a potent inhibitor of eukaryotic protein synthesis (Sasse et al., 1995), while the actions of the remaining natural products are not known. On the other hand, the biological activities of the compounds selected from the synthetic library were herein described for the first time and, for that reason, their action modes were not known yet.

To confirm the selectivity and specificity of the new RCL to detect compounds that function as inhibitors or enhancers of IFN-I activity, the effect of the 15 selected compounds was studied using a murine RCL (NIH3T3 IRF7-mCherry). In these cells, the IRF7 promoter is activated after murine IFNs-I incubation and consequently, mCherry expression is induced (Rand et al., 2012). By means of this system, Tub I plus IFN-I-treated cells showed a higher percentage of mCherry expression than cells treated only with IFN-I while the inhibitor compounds did not evidence mCherry expression (data not shown). Because NIH3T3 IRF7-mCherry is a murine model and the promoter IRF7 is an ISG different to Mx2, the effects of the compounds on the new human RCL were validated, allowing to confirm their modulator action on IFN-I activity. In addition, the ability of these compounds to modulate IFNs-I activity was evaluated by using another reporter system which employs a different reporter protein: luciferase instead of EGFP (Seo et al., 2009). Consequently, the same results were obtained through this RGA. A table providing this information was added as supplementary data. Finally, the effect of the selected compounds was assayed on a cell line that constitutively expresses the reporter protein EGFP with the purpose of evidencing fluorescent artifacts. None of them showed any differences in the EGFP expression levels. Therefore, different assays contributed with evidence that supported the concept of compound specificity.

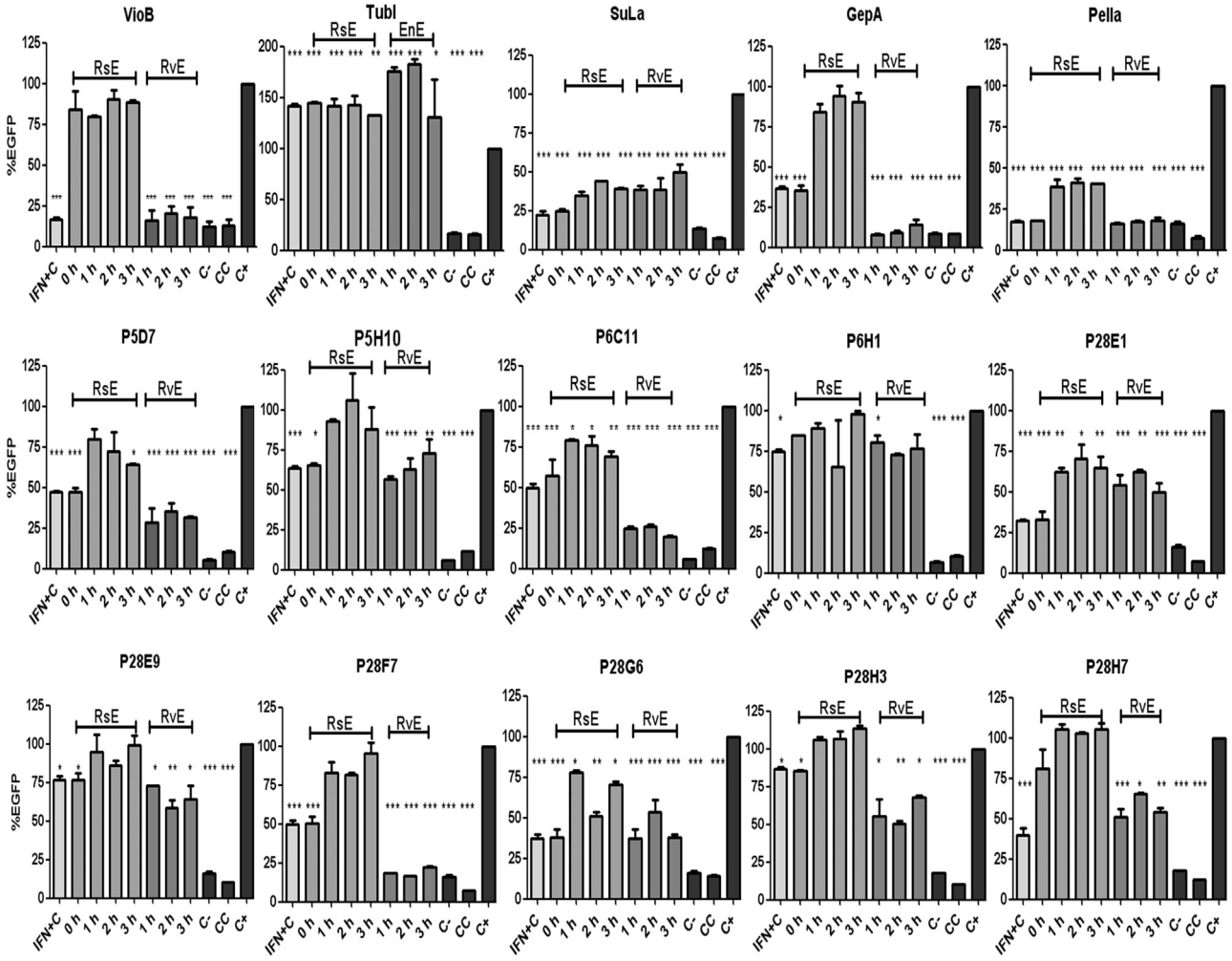


Fig. 3. Analysis of temporal action of compounds and its effect after IFN activation using the WISH cell-based RGA.
IFN + C: co-incubation control; C+: positive control; C-: negative control; CC-: compound control. RsE (residual effect): compounds were incubated for 1 h and then rhIFN-β1a (12 IU/ml) was added at 0, 1, 2 or 3 h after washing the plates. RvE (reversal effect) and EnE (enhanced effect): after rhIFN-β1a (12 IU/ml) incubation during 1, 2 or 3 h, compounds were added at their mCHRs without removing the cytokine. After treatments, the%EGFP was determined and plotted as a function of the conditions tested (horizontal axis).
*, **,***: statistics indicate the degree of significance in relation to the positive control assay, corresponding to $p < 0.1$; $p < 0.01$ and $p < 0.001$, respectively. ANOVA statistical test followed by Tukeyís post-hoc test was applied.

3.3. Compound cytotoxicity and mCHR evaluation

The cytotoxicity and mCHR of the "active" compounds, the 15 primarily identified as hits, were evaluated. Toxicity is the inherent capacity of each chemical to produce negative effects in cell cultures and consequently in living organisms. The toxicity of each compound should be determined early and particularly for each cell line, given that cell susceptibly could be different according to the cell line characteristics. In order to determine the non toxic limit concentration, each RCL was incubated with serial dilutions of compounds and compared with non-treated cell cultures (negative control). Thus, the non-toxic limit concentration was calculated as the maximum concentration of compound which produced the same color intensity as the negative control. Only TubI showed a toxic effect at concentrations higher than 10 μM, while the remaining compounds did not show toxic effects for the studied dilutions.

Additionally, the mCHR of each selected compound was studied evaluating seven two-fold serial dilutions of each compound for the four RGAs. The mCHR was calculated as the minimal concentration of each compound that produces the highest response, that is to say, the enhancement effect (above $\mu_{pos} + 3\sigma$) or inhibition effect (beyond $\mu_{pos} - 3\sigma$) produced on IFN activity.

All the synthetic compounds demonstrated the highest modulator effect at the same concentration of 12.5 μM. Meanwhile the mCHRs from the natural compounds were different (VioB: 900 μM; TubI: 10 μM; SuLa and Pella: 1,000 μM; GepA: 400 μM), with TubI exhibiting a rather low concentration to obtain the optimal response.

Taking into consideration the complete set of selected compounds, only TubI was able to increase the rhIFNs activity while the others decreased or inhibited it.

3.4. Characterization of the hits

3.4.1. Analysis of their temporal action using the WISH cell-derived RGA

The temporal action of compounds was analyzed taking into account their residual effects after removing them from the cell culture medium. To accomplish that, IFN was added at different times after 1 h compound incubation and removal, and the residual effect duration was investigated. This study allows hypothesizing about

A

Compound	Phases	CWT	rhIFN-α2a	rhIFN-β1a
Without compound (control)	SubG1	5	6	8
	G0/G1	72	63	47
	S	17	25	41
	G2/M	6	6	4
	Phases	**CC**	**rhIFN-α2a**	**rhIFN-β1a**
Tub I	SubG1	8	8	7
	G0/G1	2	8	4
	S	20	32	41
	G2/M	70	52	48
P6C11	SubG1	19	45	36
	G0/G1	65	39	49
	S	13	14	14
	G2/M	3	2	1
P28F7	SubG1	8	7	13
	G0/G1	72	74	68
	S	13	13	12
	G2/M	7	6	7
P28H7	SubG1	4	7	31
	G0/G1	71	64	47
	S	17	22	21
	G2/M	8	7	1

B

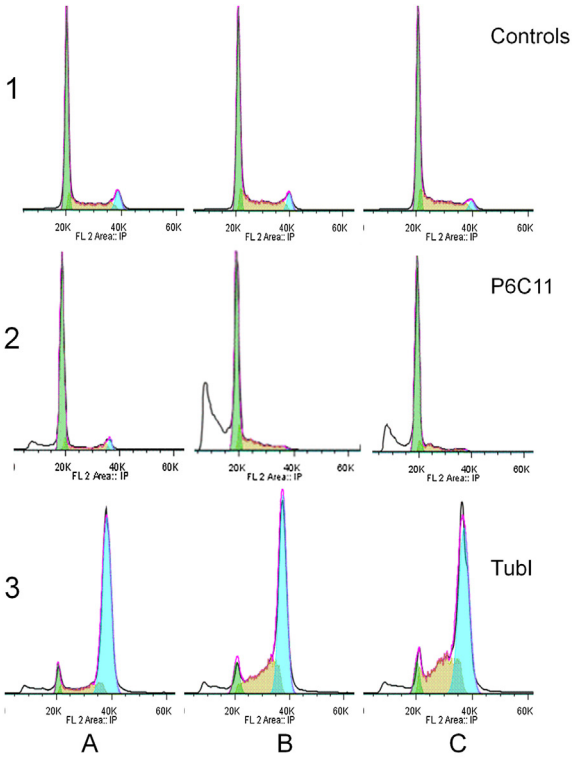


Fig. 4. Effect of natural and synthetic selected compounds on cell cycle phase distribution.
Left panel: The behavior from Tub I, P6C11, P28F7 and P28H7 was analyzed over the cell cycle phases by flow cytometry with FlowJo cell cycle analysis software application. The percentage of cells in each cell cycle phase (Sub G1, G0/G1, S, G2/M) was calculated from every assayed condition and compared. CWT: cells without treatment, CC: compound control, rhIFN-α2a or rhIFN-β1a: only rhIFN treatments, rhIFN-α2a/rhIFN-β1a plus TubI, P6C11, P28F7 or P28H7; compound plus rhIFN treatment.
Right panel: The effect of P6C11 (synthetic compound) and TubI (natural compound) on the cell cycle of WISH-Mx2/EGFP cells was analyzed in combination with rhIFN-α2a and rhIFN-β1a, and compared to the corresponding controls.
Histogram plot represents the frequencies (%) of each cell cycle phase (sub G1 peak, G1, S, G2/M) at the studied conditions. A1: Control without treatment; B1: rhIFN-α2a control; C1: rhIFN-β1a control; A2 and A3: compound controls; B2 and B3: compound + rhIFN-α2a; C2 and C3: compound + rhIFN-β1a.
Statistical analysis was performed by ANOVA test followed by Tukeyís test to determine significant differences between samples and controls. Data was analyzed using FlowJo software, making adjustments to Watson's model (Pragmatic).

the mode of action of the compound and its behavior in further *in vivo* analysis.

All selected natural compounds, with exception of VioB, maintained their effects on IFN activity after their removal from the incubation media. However, they showed different residual activity, e.g., TubI, Sula and Pella exhibited the highest residual response, prolonging their effect even when the rhIFN was added 3 h after removing the compound (see Fig. 3, at 3 h). This result suggests that these compounds would preserve their effects on IFNs activity over time. From the synthetic compounds, different activities could be found. P28E1 and P28G6 showed a long-lasting inhibitory effect on IFN activity like Sula and Pella. P28H3 and P28H7 did not present residual effects, just like VioB. The rest of the synthetic compounds exhibited residual effect only for some conditions and not as strong as the one exerted by P28E1 or P28G6.

In summary, most of the compounds had the property to preserve their modulating action on IFN activity despite removal from the culture media. Also, natural compounds exhibited stronger actions than synthetic compounds (Fig. 3). These results confirm the persistence and stability of the compounds enhancing and inhibitory effects in order to determine the best conditions to be used in future applications.

Additionally, it is worth noting that a 1 h incubation period is enough to achieve the modulator effect of the compounds on IFN activity.

3.4.2. Analysis of compound effect after IFN activation using the WISH cell-derived RGA

Specific binding between IFN and its receptor in the cell surface results in activation of STAT proteins through their phophorylation which is necessary to constitute a complex of proteins able to penetrate the nucleus and associate with regions of genes that initiate or enhance gene transcription: ISRE (IFN-sensitive response elements) (Billiau, 2006). Through the induction of a set of immediate-early response genes, like the Mx2 promoter, IFNs mediate their biological response (Mowen and David, 2000). Considering that information, the ability of inhibitor/enhancer compounds to reverse/augment the biological activity of the cytokine was evaluated using WISH-Mx2/EGFP RGA after triggering the cytokine action for 1, 2, or 3 h.

Natural compounds: VioB, Sula, GepA and Pella, as well as synthetic compounds: P5D7, P6C11, P28E1, P28E9, P28F7, P28G6, and P28H7 showed the capacity to revert the IFN activation pathway. Providing that all these compounds are inhibitors of IFN activity, the effect of reversal was observed as a reduced EGFP expression.

Furthermore, the reverse action was truly significant considering that IFN was not removed from the cultures, and that the effect

was observed even when the compounds were added 3 h after IFN incubation (Fig. 3–RvE action).

Particularly, TubI demonstrated a higher enhancement action when it was added 1 and 2 h later than the cytokine (Fig. 3–EnE action).

The remaining synthetic compounds (P6H1 and P28H3) also showed the capacity to revert the activated IFN pathway; however, effect was weaker than that of the above mentioned compounds. Therefore, this analysis allowed us to demonstrate that all inhibitor compounds were able to block IFN signaling in a cellular system that had adjusted to the harsh effects of IFN induction, and provided evidence of the ability of these molecules to reverse IFN-induced effects. Also, in the case of the enhancer molecule, the action of IFN was notably increased.

3.4.3. Compound effects on hIFN-I antiviral and antiproliferative activity

IFNs-I (mainly IFNs α/β) are essential anti-viral cytokines produced in response to the detection of viral components by host pattern recognition receptors (Lopez and Hermesh, 2011). This is why the biological activity of IFNs is often quantified by an AVA based on its capability of inducing an antiviral state in target cells (Kugel et al., 2011; Meager, 2003).

IFNs-I are also important cytokines because of their antiproliferative activity, they inhibit the proliferation of both diploid and transformed human cells *in vitro* (Borden et al., 1982).

To further validate the effects of compounds selected by RGA on hIFN-I biological activity, each of the 15 primary hits was subjected to AVA and APA procedures using the WISH-Mx2/EGFP and the parental WISH cell lines. As no differences were observed during quantitation of IFN activity by AVA or APA procedures using the parental or reporter cell lines (Bürgi et al., 2012), the following results are shown considering the experiments using WISH-Mx2/EGFP. Thus, dose-response activities of hIFNs-I (α and β) were measured in the presence of the mCHRs of all 15 compounds (Table 2).

The biological action of compounds on IFN antiviral activity was evaluated in AVA experiments. The N* value was calculated for each compound. N* represents the ratio between the antiviral activity of rhIFNs plus a given compound and the antiviral activity of rhIFNs control. Those compounds showing an N* value higher than 1 represent an antiviral increasing response while those exhibiting an N* value lower than 1 have an antiviral decreasing response. The compounds VioB, GepA, P5D7, P6C11, P28E1, P28F7 and P28G6 showed the same behavior demonstrated by RGA, decreasing the antiviral activity of both rhIFNs. The remaining compounds did not show any effect on AVA.

TubI showed a particular response. Although it was classified by RGA as an enhancer of IFN activity, it showed an inhibitory action during AVA. This was evidenced when TubI-treated cells were analyzed by microscopy. Detached cells that preserved a good viability were observed. TubI obviously affected structures involved in the surface adhesion and, as a consequence, cells detached from the substrate showing similar morphology to suspension cells. This might be connected to the well-known disrupting effect of tubulysin on the microtubular cytoskeleton (Sasse et al., 2000).

The biological action of compounds on IFN antiproliferative activity was evaluated in APA experiments. Table 2 shows the effect of each compound on APA expressed as an increase, a decrease, or having a non-significant effect (NSE). The N* value was calculated for each compound, N* represents the ratio between the antiproliferative activity of a given compound plus rhIFNs and the antiproliferative activity of rhIFNs control. Compounds showing an N* value higher than 1 represent a proliferation increasing response while those exhibiting an N* value lower than 1 have a proliferation decreasing response. An enhancing response was shown by TubI. The opposite effect was observed for VioB, GepA, Pella, P6C11, and P28H3. These results are consistent with the results obtained by RGA and AVA. Interestingly, some compounds showed NSE on proliferation, while inhibiting RGA. The discrepancy between the RGA, AVA and APA could be attributed mainly to different mechanisms of action of those compounds that make them potentially worthy for the modulation of *in vivo* IFN activity, e.g. GepA.

3.4.4. Action of compounds on cell cycle

The effect of selected compounds on cell cycle phase distribution was analyzed using the WISH-Mx2/EGFP cell line. Cell cycle phase distribution (G1, G2-M and S) was obtained as mentioned in the Materials and methods section. In addition, the subG1 peak was analyzed as an indicator of possible apoptosis. Cell cycle phases obtained from cultures treated with IFN and compounds were compared with cell cycle phases obtained from cultures only treated

Table 2
Modulator effect from natural and synthetic compounds on IFN activity measured by RGA, APA and AVA assays.

*N**							
	Name	RGA		AVA		APA	
		rhIFN-α2a	rhIFN-β1a	rhIFN-α2a	rhIFN-β1a	rhIFN-α2a	rhIFN-β1a
Natural compounds	VioB	0.1	0.1	0.6	0.5	0.6	0.6
	TubI	1.5	1.4	ND	ND	NSE	1.4
	Sula	0.6	0.5	NSE	NSE	NSE	NSE
	GepA	0.1	0.1	0.4	0.3	0.9	NSE
	Pella	0.5	0.4	NSE	NSE	NSE	0.6
Synthetic compounds	P5D7	0.7	0.7	0.9	0.8	NSE	NSE
	P5H10	0.9	0.9	NSE	NSE	NSE	NSE
	P6H1	0.9	0.9	NSE	NSE	NSE	NSE
	P6C11	0.6	0.5	0.7	0.7	0.9	NSE
	P28E1	0.8	0.7	0.9	0.9	NSE	NSE
	P28E9	0.9	0.7	NSE	NSE	NSE	NSE
	P28F7	0.7	0.5	0.7	0.6	NSE	NSE
	P28G6	0.7	0.6	0.7	0.6	NSE	NSE
	P28H3	0.8	0.8	NSE	NSE	0.8	0.8
	P28H7	0.9	0.9	NSE	NSE	NSE	NSE

The responses obtained from the compounds plus IFN and the response from the IFN control were compared in every case. The N* value was calculated as follows:
N*: activity (RGA/AVA/APA) ratio of a given compound plus rhIFN-α2a or rhIFN-β1a and the activity (RGA/AVA/APA) of rhIFN-α2a or rhIFN-β1a control.
Values higher than 1 represent an increase of the IFN biological activity and values lower than 1 account for a decrease of it.
ND: not determined.
The effect of TubI on IFN activity measured by AVA could not be determined through this assay because this compound detached the cells from the substrate.

with IFN (IFNs control), cultures only treated with each compound (CC–compound control) and cultures without treatment (CWT–negative control).

Compounds P5D7, P6H1, P28E1 and P28E9 did not alter the cell cycle (data not shown). Other compounds, such as VioB, SuLa, GepA, Pella, P5H10, P28G6 and P28H3, showed differences with respect to the controls, but they did not exhibit differences when they were co-incubated with IFNs. Interestingly, TubI, P6C11, P28F7 and P28H7 exhibited differences when they were compared to IFNs controls but also when the correlation was made between the compound control and cultures treated with IFN plus the corresponding compound. Fig. 4A summarizes the observed effects. The combination of P6C11 with both IFNs caused a decrease in the percentage of cells in each phase of the cycle, at the expense of an increased subG1 peak which is indicative of an apoptotic process (Fig. 4B). The most striking effect was induced by TubI, an identified inhibitor of microtubule polymerization (Khalil et al., 2006). As recently described by Murray et al., 2015; Tubulysins are a family of antimitotic tetrapeptides isolated from myxobacteria reported by Sasse and co-workers in 2000. They have potent antiproliferative activity against human cancer cells, including drug-resistant cells. It was quickly established that the mechanism of action of tubulysins is the inhibition of the polymerization of the cytoskeletal protein tubulin and the induction of apoptosis. A G2/M delay is typical for compounds that interfere with spindle formation (Fig. 4B). Therefore, since IFNs have antiproliferative and anti-tumoral activity, it is likely that Tub might act in combination with type I IFNs to potentiate antiproliferative and antitumoral responses. In this work, the combination of TubI with rhIFN-α2a or rhIFN-β1a was analyzed using different assays as antiproliferative activity assay and cell cycle analyses, demonstrating the above-mentioned potentiation effect. In contrast, P28H7 showed differences only when cells were treated in combination with rhIFN-β1a, reducing the percentage of cells in S and G2-M phases, while augmenting the subG1 proportion (31%). Similar results were obtained when cells were exposed to P28F7 but the increase in the subG1 peak was not so pronounced (13%).

Analyzing the results herein obtained for TubI, P6C11, P28F7 and P28H7 and comparing them to those obtained by RGA, AVA and APA assays (Table 2) it could be evidenced that these compounds were identified as modulators of IFN activity through RGA. Nevertheless, these compounds showed different responses when they were studied by AVA and APA assays. In the case of TubI, its effect on IFN activity was not well evidenced by AVA because this compound acted in cells detaching from the culture surface and, thus, the assay could not be carried out. When P6C11 was investigated by APA, a NSE was observed when it was co-incubated with rhIFN-α2a or rhIFN-β1a. Finally, neither P28F7 nor P28H7 showed any effect on APA when combined with IFN. Furthermore, P28H7 showed NSE on IFN activity on AVA.

Considering the above results, TubI, P6C11, P28F7 and P28H7 compounds that were identified by RGA and later by cell cycle analysis, would have been discarded if the screening had been carried out with the usual assays (APA and/or AVA).

4. Conclusions

Considering that IFNs are central molecules with pharmacological or pathological actions that have to be differentially managed, this work provides the foundation to understand how to proceed in the selection of compounds that could be useful either to improve or to overcome the above mentioned effects.

Therefore, novel RGAs that had been previously developed were satisfactorily used as biological tools to screen and characterize several compounds from natural and synthetic libraries. These assays gave consistent results in a short period of time using an excellent and safer methodology than those routinely used, such as the AVA procedures.

Therefore, by using RGAs in a LTS approach with 442 compounds, some promising candidates that inhibit rhIFN activity were found. Two of them were from natural origin (VioB and GepA) and three were from synthetic origin (P6C11, P28F7 and P28H7).

Furthermore, TubI, a compound that interferes with microtubule formation was identified as a candidate that enhances rhIFN activity. In addition, its positive effect is long-lasting, suggesting that TubI acts as a primer. Such priming is well known in the IFN system. Primers induce one of the key signaling compounds or change epigenetic behavior in the cell, and consequently induce a long-lasting action on IFN-enhancing effect.

In conclusion, the IFN-I enhancer or IFN-I inhibitors described herein are valuable starting points to design more potent derivatives in order to obtain highly active substances and modulate the dual clinic effect of these cytokines.

Acknowledgements

This project was funded by FOCEM (MERCOSUR Structural Convergence Fund), COF 03/11. Milagros Bürgi would like to acknowledge the financial support provided by CONICET.

We would like to thank Valentina Porro for the assistance during cell cycle analysis. The authors would also like to thank Gisela Leal from the Cell Culture Laboratory at Universidad Nacional del Litoral for meticulously proofreading the manuscript, and Dr. G. Dräger from Leibniz University of Hannover, for HRMS measurements.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2016.06.021.

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