A COMPARISON OF THE BINDING OF INTERFERON ALPHA SUBTYPES TO ISOLATED IFNAR1 AND IFNAR2 WITH ACTIVITY IN ANTIVIRAL AND ANTIPROLIFERATIVE ASSAYS.

Thomas B. Lavoie[1], Eyal Kalie[2], Renne Abramovich[2], Sara Crisafulli Cabatu[1], Gina DiGioia[1], Karlene Moolchan[1], Sidney Pestka[1], Gideon Schreiber[2]

[1]PBL InterferonSource, 131 Ethel Rd West, Piscataway, NJ 08854 USA, [2]Weitzmann Institute of Science, Rehovot, Israel.

Abstract:

Interferon alpha (IFN-α) subtypes have long been understood to have differing activities in a variety of assay systems. In order to better understand the determined the binding to isolated IFNAR1 and IFNAR2 extracellular domains by surface plasmon resonance. We observed a 10-fold difference in binding to IFNAR1 with IFN-α8 binding tightest and IFN-α17 binding weakest. Binding to IFNAR2 varied by nearly 100-fold with IFN-α10 binding tightest and IFN-α1 binding weakest. IFN bioactivity was determined by protection against Encephalomyocarditis virus on A549 and OVCAR-3 cells, Vesicular stomatitis virus on A549 and WISH cells, and antiproliferative activity on WISH and OVCAR-3 cells. In general, the binding related well to activity in these systems with some exceptions. IFN-α16 and IFN-α14 generally exhibited potent antiproliferative activity but not antiviral activity. Other subtypes, such as IFN-α8 and IFN-α10, were highly potent in some systems but not in others. A variety of mathematical models were tested to predict activity from binding, and several gave reasonable fit to the data with the most frequent exceptions being IFN-α6 and IFNα7. These results suggest that while binding is the primary driver of activity, additional variables will need to be considered to explain differences in activity based on factors, such as cell type, challenge virus, and assay format.

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Materials and Methods.

Cells and reagents.

The lung epithelial carcinoma A549 (CCL-185) and the ovarian carcinoma OVCAR-3 (HTB-161) were obtained from ATCC. A549 was grown in DMEM (Gibco) with 10% FBS (Gibco).. OVCAR-3 was grown in RPMI-1640 with 10% FBS. WISH cells were obtained from Dr. Daniels Novick, (Weizmann Institute). Encephalomyocarditis virus (EMCV ATCC-VR129B) and vesicular stomatitis virus (VSV ATCC-VS158) stocks were produced on Vero cells. Bulk interferons from PBL Biomedical Labs included Alpha1 (11175), Alpha2a (11100), Alpha2b (11105), Alpha4b (11180), Alpha5 (11135), Alpha6 (11165), Alpha7 (11160), Alpha8 (11115), Alpha10 (11120), Alpha14 (11145), Alpha16 (11190), Alpha 17 (11150), Alpha 21 (11130) and AlphaD (11125). All these molecules were expressed in E. coli as inclusion bodies, solubilized with guanidine-HCl and refolded by dilution. Purification was by a combination of hydrophobic interaction chromatography, size exclusion chromatography and ion exchange chromatography. In all cases purity was greater than 95% with <1 endotoxin unit/ml. Endotoxin was determined either by LAL or fluorescent assays (Cambrex) as recommended by the manufacturer.

In vitro binding assay.

Binding affinities of IFN α 2 toward IFNAR1-EC or IFNAR2-EC (extracellular domain) were measured using the ProteOn XPR36 Protein Interaction Array system (Bio-Rad), based on surface plasmon resonance technology. PBS was used as running buffer at a flow rate of 30 µl/min. For immobilization of the IFNAR1 receptor subunit, a NeutrAvidin-covered sensor chip (NLC, Bio-Rad) was bound with 150 nM biotin-conjugated trisnitrilotriacetic acid (BTris-NTA) (Reichel, A. et al. 2007). The IFNAR1-EC subunit was then injected to separate channels in duplicates, at 100 nM and 200 nM respectively, to bind the tris-NTA on the chip surface via their His $_6$-tags. The IFNAR2 receptor was immobilized as detailed in Kalie, E. et al.2007. The tested interferons were then injected perpendicular to ligands, at six different concentrations within a range of 16.5 to 4,000 nM for IFNAR1 binding and 6.25 to 200 nM for IFNAR2 binding. Data were analyzed using BIAeval 4.1 software, using the standard Langmuir models for fitting kinetic data. Dissociation constants KD were determined from the rate constants according to

$$K_D = \frac{k_c}{k_a} \quad \text{(eq. 1)}$$

or from the equilibrium response at six different analyte concentrations, fitted to the mass-action equation (Pichler, J. and Schreiber, G. 1999).

Antiviral assays.

For the A549 and OVCAR-3 assays 0.1 ml of 1X105 and 3X105 cells/ml solution, respectively, were added to the wells of a microtiter plate and the cells were then place in a 37°C/5% CO2 incubator for 2-4 hours. Serial dilutions of IFN were prepared by two fold dilution across another microtiter plate, and 0.1 ml of the dilutions were added to the plated cells. All titrations were calibrated to a lab standard of IFN-alpha2a which in turn had been calibrated to the reference standard for IFN-alpha (Gxa01-901-535). The IFN was allowed to interact with the cells for 18-24 hours in the incubator. At this point 0.05 ml of a dilution of EMCV or VSV was added which had been empirically determined to kill >95% of the cells in 40 hours. After 40-48 hours of incubation the plate was stained with crystal violet and excess dye washed out with cold tap water. After drying the dye was solubilized with 70% MeOH and the absorbance at 570 nm was determined using a Molecular Devices plate reader. Data was analyzed in Graphpad Prism using a variable slope sigmoidal curve fit using a cell control (No IFN, no virus) as 100% and a virus control (No IFN, with virus) as 0%. EC50 was determined relative to the Alpha2a standard. Titrations were done in duplicate and repeated on at least 3 independent occasions.

Antiproliferative assays.

For the OVCAR-3 AP assay 0.1 ml of 1X104 cell/ml solution was added to a microtiter plate. Serial dilutions of the different IFN-alphas were prepared as in the AV assays and after 2-4 hour were added to the cells. The plates were then incubated for 5 days and the cell number determined by MTS (Promega) assay system. Data was analyzed as for the AV assays using a cell control (Cells no IFN) as 100% and media as 0%.

For WISH cells the antiviral and antiproliferative assays were performed as described in Kalie, et al. 2008. In summary, WISH cells were plated in flat-bottomed microtiter plates and interferon was added at serial dilutions. In the antiproliferative assy, cell density was monitored after 72 h by staining with crystal violet from which the 50% activity concentrations (EC50) was calculated. Antiviral activity was assayed as the inhibition of the cytopathic effect of vesicular stomatitis virus on human WISH cells. Four hours after the addition of interferon, vesicular stomatitis virus was added to all wells, and after 17 h of incubation cell density was measured by crystal violet staining. EC50 was calculated by fitting the absorption of the crystal violet stain. Both the antiviral and antiproliferative assays were repeated at least 3 times for each protein. The experimental error (σ) for both assays was 35%. Therefore, a confidence level of 2σSE would suggest that differences smaller than 2-fold between interferons are within the experimental error.

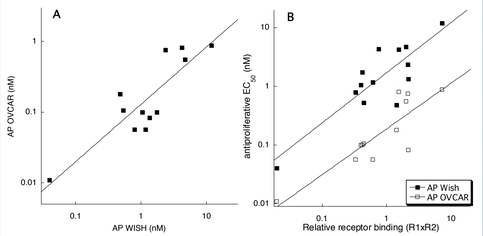
Table 1. Kinetic and Affinity Measurments of IFN-Alpha Subtypes.

	R1* K_D (µM)	R2* k_d (x10^6 $M^{-1}s^{-1}$)	R2** k_a (x10^6 $M^{-1}s^{-1}$)	R2 k_d (x10^{-3} s^{-1})	R2* K_D (nM)	R2** K_D (nM)
A2a	3.5	4.6	ND	7.7	1.7	ND
A2b	3.8	5.5	11	7.3	1.3	0.66
A1	0.76		ND	ND	220.0	ND
A4	3	4.0	ND	6.1	2.1	ND
A5	0.94	7.4	9	27.0	3.8	3.00
A6	0.83	10.7	50	6.2	0.6	0.12
A7	1.3	9.1	24	6.1	0.7	0.25
A8	0.53	6.4	10	9.9	1.6	0.59
A10	4.3	4.5	23	1.7	0.4	0.08
A14	0.68	3.2	ND	2.3	0.7	ND
A16	1.1	4.6	11	5.1	1.1	0.46
A17	5.0	7.7	20	3.0	0.4	0.15
A21	1.25	5.4	ND	27.0	5.0	ND
A2a[#]	2.0	2.8	10	5.6	2.0	0.56

There is a >100 fold range of binding to IFNAR2 with all but A1 falling within a 12 fold range. A10, A6, A17 and A14 all bind well. There is a 16 fold range of binding to IFNAR1. Here A1 binds tightly. A8 and A14 also bind tightly while only A4 is weaker than A2a or A2b.

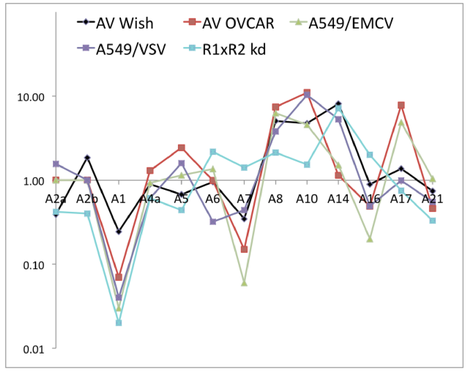
Table 2. Relative antiproliferative and Antiviral potency of Human IFN-Alpha Subtypes

Subtype	AP Wish	AP OVCAR	WISH/ VSV	OVCAR/ EMCV	A549/ EMCV	A549/ VSV
A2a	1.75	1.00	0.39	1.00	1.00	1.56
A2b	1.05	1	2.08	1	1	1
A1	0.04	0.11	0.24	0.07	0.03	0.04
A4	1.17	0.57	0.89	1.30	0.93	0.61
A5	0.53	1.05	0.68	2.43	1.14	1.59
A6	1.34	0.83	0.95	0.99	1.36	0.32
A7	0.48	1.80	0.35	0.15	0.06	0.44
A8	2.35	7.55	5.06	7.40	6.26	3.79
A10	4.22	8.12	4.02	11.01	4.56	10.32
A14	11.95	8.84	8.10	1.14	1.51	5.29
A16	4.71	5.59	0.89	0.49	0.20	0.49
A17	4.35	0.06	1.37	7.80	4.87	1.72
A21	0.79	0.57	0.74	0.46	1.04	0.55
A2b*	1.0	ND	1.0	ND	ND	ND
Beta	43	14.7	2.0	ND	0.89	ND
A2b*	1.0	ND	1.0	ND	ND	ND

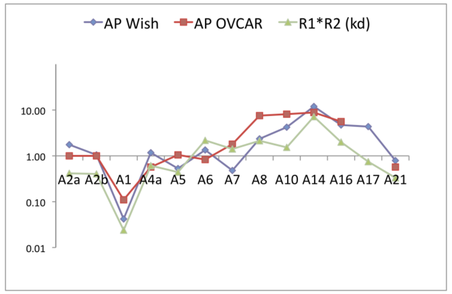


There is reasonable correlation between the two antiproliferative assays and between these assays and the receptor binding product. Notably the OVCAR cell require less interferon to observe the AP effect.

Comparison of the AV activities in various assays

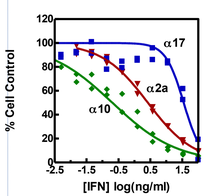


Graphic Comparison of Antiproliferative Results

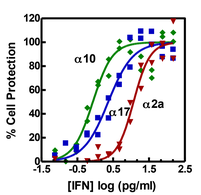


When comparing the antiviral and antiproliferative results, some interesting anomalies are observed. In all four AV assays, A16 appears less potent than A2b, whereas in both AP assays (and for R1xR2), A16 appears more potent than A2b. A14 was similar or slightly less potent at AV in the OVCAR and A549 assays, while A14 was much more potent in AP activity in the OVCAR and WISH, and more potent in the A549/VSV antiviral model. A17 was generally potent as AV and AP except for AP on OVCAR where it is significantly less potent than A2b.

AP Assay

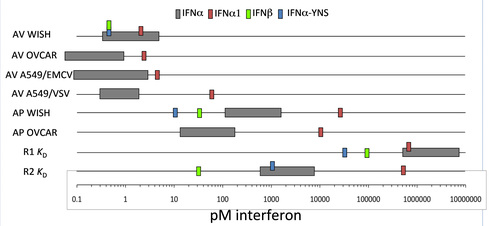


AV Assay



A17 was generally potent at AV and AP except for AP on OVCAR where it is significantly weaker than A2b

Range of Activities of Various Type I IFN Proteins In Antiviral and Antiproliferative Assays



The picomolar concenctration for the EC50 of the IFN-α subtypes, with the exclusion of Alpha-1 are shown in the grey shaded box. Alpha 1 is shown in Red. IFN-β is shown in Green and the Alpha-2 mutant YNS in blue.

In this study, we have examined all the interferon alpha protein subtypes for Type I IFN receptor subunit binding affinity as well as potency in several bioactivity assays. Overall, we observe a good correlation between binding and bioactivity. However, some significant deviations are apparent. Such deviations may provide insight into the therapeutic potential of certain interferon alpha subtypes or of modified interferon alpha subtype molecules.