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REVIEW

Russian Experience in Screening, Analysis, and Clinical Application of Novel Interferon Inducers

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ABSTRACT

This review describes a long-standing experience of screening for interferon (IFN) inducers in Russia. IFN inducers represent a special group of potential antiviral compounds. The main requirements for them are (1) high IFN-inducing activity, (2) absence of side effects, (3) wide spectrum of antimicrobial activity, (4) broad therapeutic security and, (5) good solubility in water and biologic liquids. IFN inducers stimulate IFN production in different cells and organs, and that determines the strategy for their application. Amixin (OOO "Lancepharm," Moscow, Russia) induces IFN-α/β production mostly in T cells. Cycloferon (NTFF "Polysan," St. Petersburg, Russia) stimulates B cells and macrophages to produce almost pure IFN-α. Double-stranded RNA (dsRNA) and polyphenols of natural origin stimulate IFN production in different populations of immunocytes. Only polymers, such as Larifan (Riga, Latvia), Kagocel ("NIARnedicplus," Moscow, Russia), and Ragosin (N.F. Gamaleya Institute, Moscow, Russia), induce IFN synthesis in muscles, so they may be effective against rabies. Cycloferon, Larifan, and Kagocel, which induce IFN formation in lungs, may be effective against influenza and rhinoviral infections. Cycloferon and Larifan stimulate IFN production in liver and spleen and may be effective against hepatitis B. Oral compounds (Amixin, Kagocel) that stimulate IFN production in intestines may be effective against hepatitis A and enteroviral infections. Low molecular weight inducers (Amixin, Cycloferon, Kagocel) that penetrate the blood-brain barrier may be active against viral encephalitis. At present, clinical trials of IFN inducers are limited, but in the near future, IFN inducers may be used against very different infections and conditions.

INTRODUCTION

Interferons (IFN) belong to the class of soluble factors (proteins) secreted by cells, termed cytokines. They are among the most ubiquitous substances produced by organisms in response to various stimuli. IFN are a proven natural mechanism for defense against viral infections. Bearing in mind the complex nature of IFN as a biologic system, many viruses are sensitive to its action in one way or another. Currently, there are two different approaches to the use of IFN in clinical medicine: application of purified IFN (exogenous IFN treatment) and induction in the organism of endogenous IFN synthesis with exogenous IFN inducers.

Success with exogenous IFN treatment has been well documented since the early 1970s, but later attempts to use IFN inducers suffered a reversal in spite of initial encouraging results. The main limitation to the use of IFN inducers against viral infections was the relatively high toxicity exhibited by early compounds. For most of the IFN inducers, the toxic dose exceeded the optimal IFN-inducing dosage by several orders of magnitude. In addition, in the 1980s when advances in biotechnology were applied to IFN production, a practically unlimited supply of purified IFN of different types became available for clinical trials. Consequently, the necessity for and the interest in carrying out investigations with IFN inducers were diminished.

Subsequently, it has been clearly demonstrated that sometimes therapy with exogenous IFN does not lead to the desired outcome.[1] Furthermore endogenous IFN naturally produced by the organism were found to have the following advantages over exogenous IFN in clinical trials: endogenous IFN are not antigenic, their release can be regulated to prevent oversaturation with IFN, a single injection of IFN inducer can stimulate a relatively prolonged circulation of therapeutic amounts of IFN,[2] and treatment with IFN inducers does not rule out combined therapy with exogenous IFN. Such combinations, as well

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TABLE 1. CLASSIFICATION OF SELECTED IFN INDUCERS

Classification	*Most active and potential IFN inducers*
Natural compounds	
Low molecular weight polyphenols of vegetable origin	
Derivatives of gossypol	**Megasin,**[a] Kagocel, Savrats, Ragosin, Gosalidon
Polymers	
dsRNA	**Larifan, Ridostin**
Synthetic compounds	
Low molecular weight compounds	
Fluorenones	**Amixin**
Nitric bases	**Neovir, Cycloferon**
Polymers	
Polynucleotides	**Ampligen** [Poly(I)•Poly(C_{12}U)] **Poludan,** Polyguacil

[a]Compounds in clinical practice are in bold.

as combined application with other immunomodulators, have exhibited a synergistic effect in some cases.

Endogenous IFN are synthesized in different target organs and systems, and they act at defined body sites. The mechanism of their action is complex. IFN influence target cells directly through activation of antiviral proteins, resulting in an antiviral state. They inhibit cell growth and modulate specific and nonspecific immune responses, as well as interacting with other mediators of inflammation and immune responses.

It has become clear that endogenous IFN appear to be part of a complex, physiologically active biologic system, which includes neuroendocrine and immune systems.[2] They interact by maintaining homeostasis along with other soluble or cell-associated regulatory factors. These interactions have highly complex and very important biologic and pharmacologic characteristics, which should be considered in exploiting the therapeutic characteristics of IFN inducers. Their pharmacodynamics and pharmacokinetics, as well as the sites of production and the types of endogenous IFN induced, are unique to each type of IFN inducer. They depend primarily on their chemical structures and secondarily on the nature of the viral infection.[3–5]

MAIN REQUIREMENTS OF IFN INDUCERS

IFN inducers are a relatively new group of antiviral compounds with high potential. The main requirements for a compound to be an ideal IFN inducer are (1) high IFN-inducing activity, a property intrinsic to these compounds, (2) satisfying the main principle of medicine, that is, "no harm to the patient," thus having minimal side effects, (3) a wide spectrum of antimicrobial activity, (4) broad therapeutic efficacy, and (5) high solubility in water and other biologic liquids.

TABLE 2. ANTIGENIC DESIGNATION OF IFN INDUCED BY VARIOUS IFN INDUCERS

IFN inducers	*Cells producing IFN*	*Types of IFN*
Polynucleotides	T lymphocytes, B lymphocytes, macrophages,	
Ampligen	fibroblasts, neutrophils, endothelial cells	α, β
Poludan		α, β
Polyguacil		α, β
dsRNA		
Larifan		α, β
Ridostin		α, β (γ)
Aromatic hydrocarbons		
Derivatives of gossypol		
Megasin		α, β
Kagocel		α, β
Savrats		α, β (γ)
Gosalidon		α, β (γ)
Ragosin		α, β
Fluorenones		
Amixin	T lymphocytes, B lymphocytes, macrophages, neutrophils	α, β (γ)
Acridanones		
Neovir, Cycloferon	B lymphocytes, macrophages, neutrophils	α, β

Obviously, no IFN inducer conforms to all these requirements.[6] Nevertheless, IFN inducers have been sought and discovered among viral and nonviral agents as well as among different classes of chemical compounds. These agents were found to induce different levels of IFN in organisms and tissue cultures ranging from 20 to 4,000,000 IU/ml. It is not possible to determine in principle which chemical structure is more important in fulfilling the requirements. However, it is clear that it is not necessary for one compound to possess all the preferred activities. It is more important and sufficient to discover the active chemical structures among the different classes of compounds in order to establish what kind of alterations are responsible for increasing or decreasing IFN-inducing, antiviral, or toxic activities. Such information allows one to establish a strategy for screening active IFN inducers and synthesize new derivatives.[3,4,7–13]

CLASSIFICATION OF IFN INDUCERS

At present, all the known IFN inducers can be divided into two main categories: natural and synthetic. Natural inducers include double-stranded RNA (dsRNA) isolated from yeast and bacteriophages and various polyphenols extracted from plants. Synthetic inducers include aromatic hydrocarbons and polynucleotides. Both groups of inducers can be divided into low molecular and high molecular weight preparations.[14,15]

As a result of lengthy screening tests in our laboratory, 13 prospective IFN inducers have been identified among various classes of compounds of very different origin (fluorenones, gossypol derivatives, copolymers of pyran, nitric bases, synthetic polynucleotides, natural dsRNA, and others). Potential IFN inducers are listed in Table 1.

Among the synthetic preparations we have studied, particular attention has been paid to Amixin (OOO "Lancepharm," Moscow, Russia), one of the fluorenones, Cycloferon (NTFF "Polysan," St. Petersburg, Russia), and acridanone from the class of nitric bases,[4,5–12,13,16–24] and Poludan (OOO "Lancepharm") and polyguacil from polynucleotides.[7,12,23–31]

Another group of polymers, oxibenzilamine derivatives, is among the natural IFN inducers. The most active within this group are the natural polyphenols derived from gossypol—Kagocel ("NIARnedicplus," Moscow, Russia), Megasin, Ragosin, Gosalidon (all from N.F. Gamaleya Institute, Moscow, Russia), and Savrats[3,13,24,29,30,32–36]—as well as dsRNA—Larifan (Riga, Latvia) and Ridostin ("Diapharam" NOP "VECTOR," Berdsk, Russia). These have been well studied and are the compounds of greatest interest.

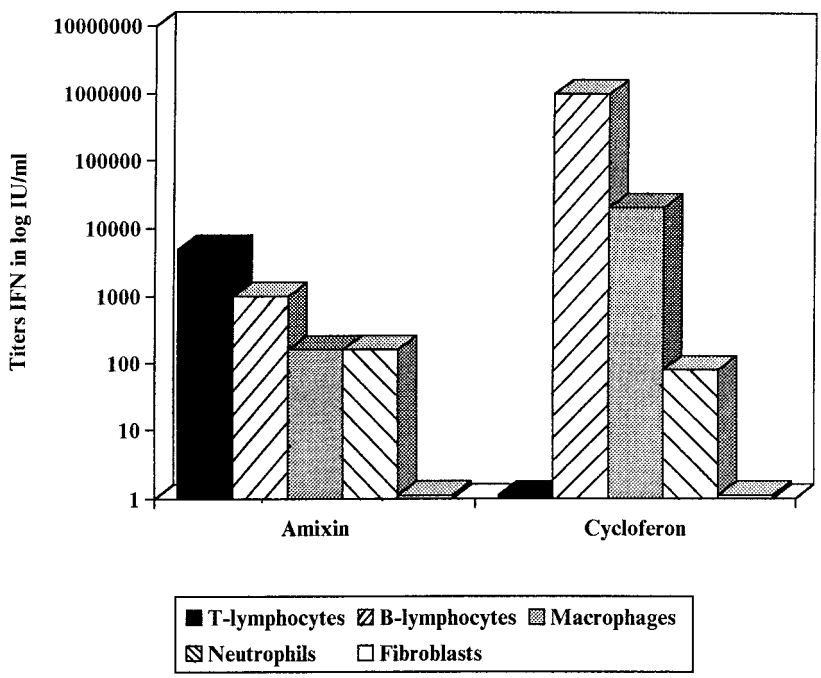

FIG. 1. Levels of IFN production by various populations of murine cells in response to induction with Amixin or Cycloferon. Spleen, blood, and peritoneal exudate were obtained from CBA mice as previously described.[13] Lymphocytes were separated from a suspension of spleen cells, and neutrophils were from peripheral blood of mice by Percoll gradient centrifugation. T and B lymphocytes were isolated by using antimouse gamma globulins (Institute of Immunology, RAMS, Moscow, Russia). Macrophages were obtained from murine peritoneal exudate by adherence. Fibroblasts were isolated by trypsinizing of mouse embryos. Amixin (200 μg/ml) and Cycloferon (600 μg/ml) were introduced to the cultures (5–10 $\times 10^6$ cells/ml) and incubated for 12, 24, and 48 h in a 5% CO_2 atmosphere. Antiviral activities of IFN were tested in L-929 cultures using encephalomyocarditis virus (EMCV) as a test virus. The maximal levels of IFN for each culture are shown in the diagram. Each point represents a mean of three or four independent experiments.

CELL PRODUCTS AND ANTIGENIC COMPOSITION OF IFN STIMULATED BY VARIOUS IFN INDUCERS

The chosen compounds combine low toxicity with the capacity to induce substantial IFN production. Various substances stimulating the synthesis of different types of IFN have been discovered. Our studies have concentrated on the prospective IFN inducers we have studied that induce IFN-α/β in various proportions (Table 2).

It should be noted that IFN-α and IFN-β exert the most definitive antiviral effects. For this reason, they are the most active stimulators of intracellular mechanisms that limit replication of viruses. In addition, type I IFN has several other actions, with those affecting the immune system being particularly prominent. IFN inducers stimulating IFN-α/β increase expression of class I MHC antigens, which are important for presentation of antigenic epitopes to various phenotypes of lymphoctyes, as well as activation of T helper (Th) cells and regulation of antibody production by B cells. These effects are significant for the development of efficient antimicrobial immunity and may also regulate the migration of cells, especially to lymphoid organs. Thus, the advantage of IFN inducers over other antiviral compounds is their wide spectrum of antimicrobial action.

Investigation of IFN inducers in our laboratory revealed that they can be distinguished from each other by certain characteristics. Thus, despite the fact that most cells are believed to be able to produce type I IFN, different phenotypes of immunocytes participate in the synthesis of IFN that are induced by various chemical compounds. The kinetics of IFN synthesized following induction, as well as their antigenic composition, also depend on the chemical structure of the inducer, the mode of its application, and the target cells stimulated by it.[3–5,10,13,22–24,26,37–39] For example, two low molecular weight hydrocarbons, Amixin (an analog of tilorone)[4,13,24,38] and Cycloferon (an analog of Kamedon)[4,5,24,38] induce IFN production in different cells (Fig. 1). Amixin stimulates IFN-α/β synthesis in different types of leukocytes, but mostly in T cells. As Amixin is a low molecular weight substance, IFN synthesis by T lymphocytes is realized without the help of other cells as presenters.

In contrast, Cycloferon induces IFN production in B cells and macrophages. Neutrophils also may participate in IFN synthesis induced by Cycloferon.[38] All these populations of cells produce IFN-α/β, but mostly IFN-α.[40–42] Consequently, a

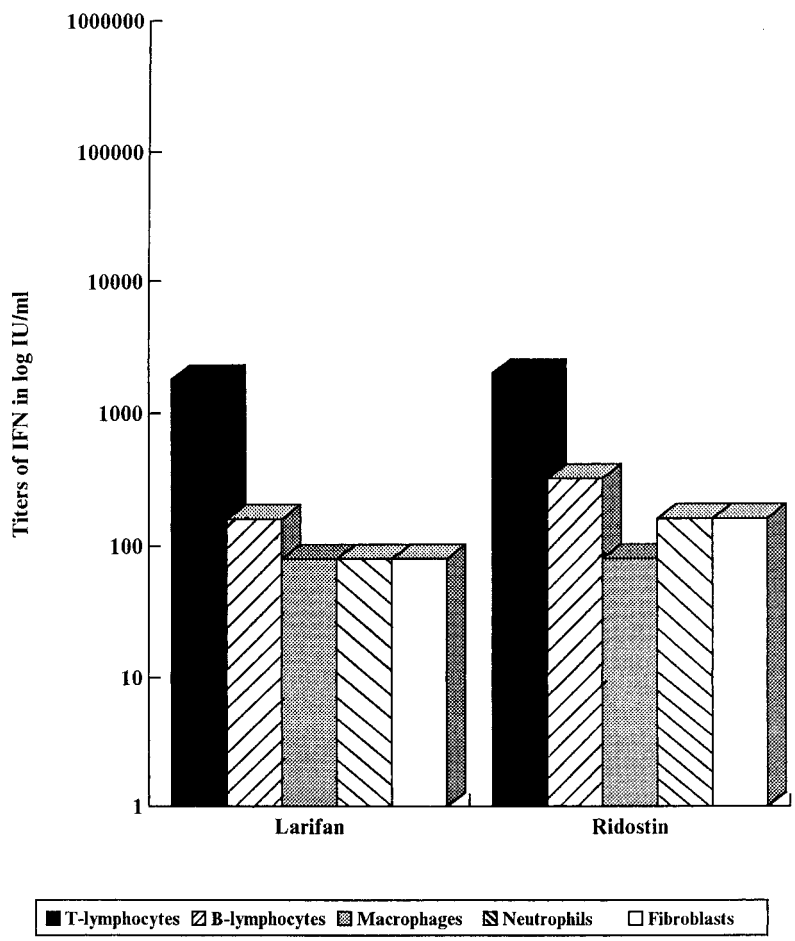

FIG. 2. Levels of IFN production by various populations of murine cells in response to induction with Larifan (25 μg/ml) or Ridostin (25 μg/ml). Conditions are as described for Figure 1.

practically pure IFN-α preparation results in response to Cycloferon.[38]

As a rule, polymers are polyclonal IFN inducers and stimulate the formation of high levels of IFN-α/β in various proportions by different populations of immunocytes, including T and B lymphocytes, macrophages, neutrophils, fibroblasts, and endothelial cells. The amount of each type of IFN depends on the degree of participation of different cell populations in its production.

Ridostin and Larifan belong to the category of dsRNA of natural origin (Fig. 2). As dsRNA are rather large molecules, IFN production induced by these compounds is carried out mostly by T lymphocytes with the help of additional cells.[23,24,39] Dendritic cells are presumed to have an inducer-presenting function in this case as it has been shown that dendritic cells are presenters of antigen to Th cells. In addition, type alpha dendritic cell precursors show an ability to produce IFN-α/β in response to viruses.[41,42]

Derivatives of gossypol (Kagocel and Ragosin) (Fig. 3) represent the other group of natural polymers. These preparations induce IFN production in several cell populations,[24,34,35] but unlike dsRNA, they are low molecular weight compounds. Thus, they induce IFN production in T cells without the help of other cells, as does Amixin.

PRODUCTION OF IFN BY DIFFERENT ORGANS AND TISSUES

Various compounds induce IFN *in vivo,* predominantly in lymphoid organs, which again suggests an immunoregulatory function. The availability of IFN in an organ determines the strategy of the inducer application.[13,24,36,38]

Figure 4 demonstrates the maximal levels of IFN production induced by various compounds in different organs of mice. IFN is synthesized in muscles only in response to induction by such polymers as Larifan, Kagocel, and Ragosin. Consequently, these polymers are used against such infections as rabies.[43,44] Low molecular weight hydrocarbons, such as Amixin and Cycloferon, do not induce IFN in muscles, and, thus, they are not effective against this infection. Some IFN inducers (Amixin, Cycloferon, Larifan, and Kagocel) have an affinity for the corresponding receptors on alveolar macrophages and induce IFN formation in lungs.[4,24,33,34] They are effective against influenza, rhinovirus, and adenovirus infections.[4,33,45–48]

Most inducers cause IFN synthesis in the liver. Those that also induce IFN in spleen (Cycloferon, Larifan) may be effective against hepatitis B. Only a few inducers stimulate IFN production in intestines. Each of these is an oral compound. They

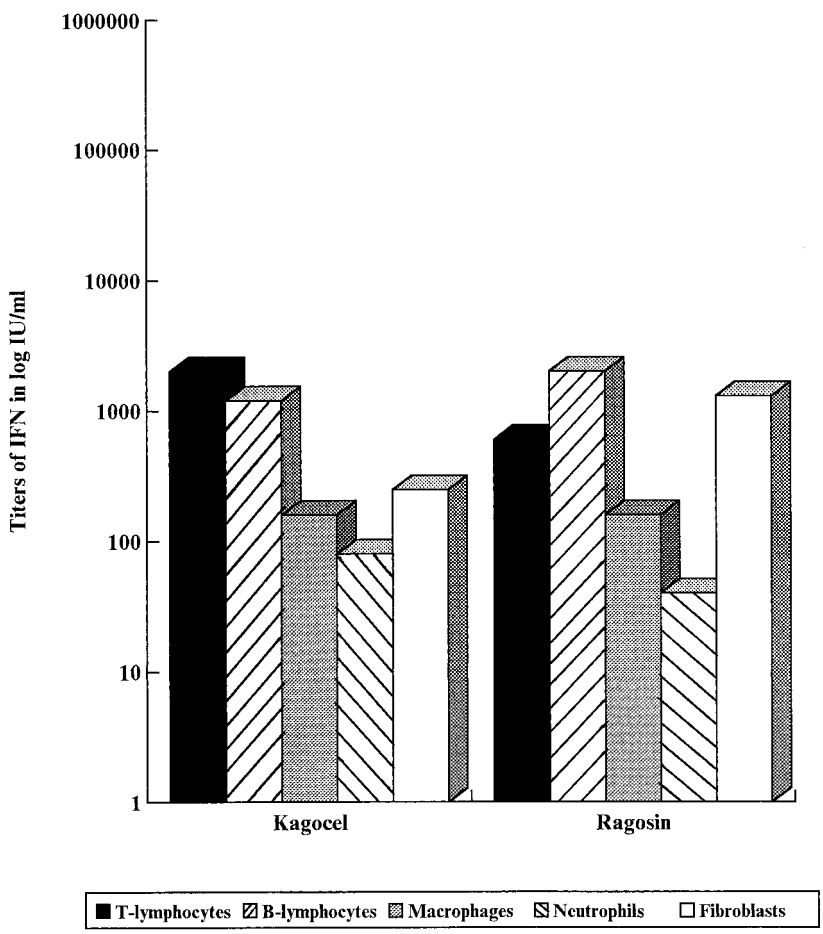

FIG. 3. Levels of IFN production by various populations of murine cells in response to induction with Kagocel (125 μg/ml) or Ragosin (125 μg/ml). Conditions are as described for Figure 1.

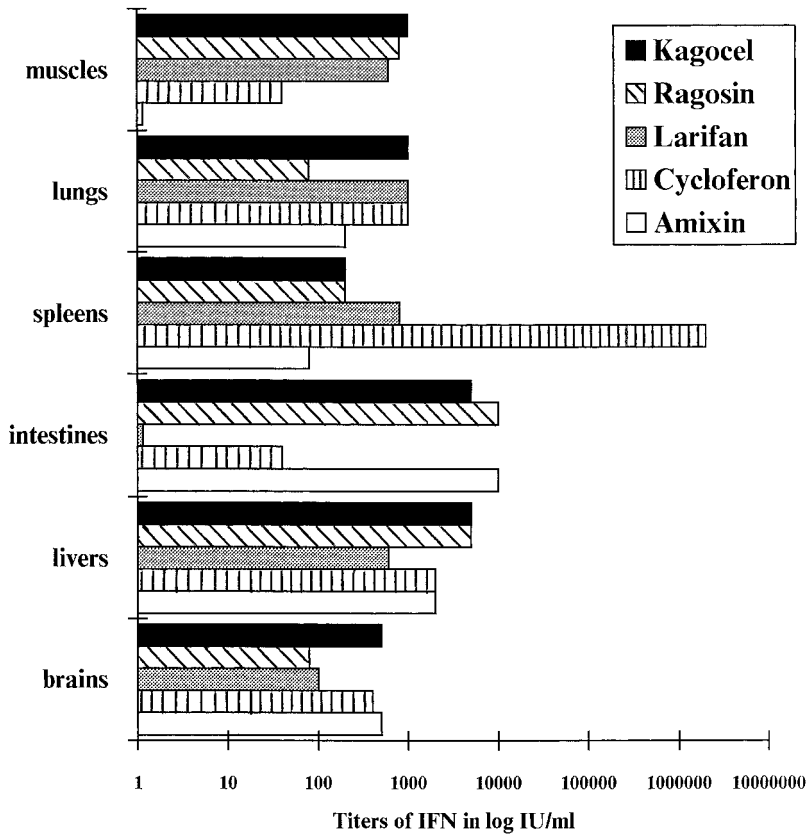

FIG. 4. The maximal IFN levels were determined in different target organs and tissues of CBA mice in response to induction with one of the following inducers: Kagocel (250 μg/mouse), Ragosin (250 μg/mice), Larifan (200 μg/mouse), Cycloferon (600 μg/mouse), and Amixin (50 μg/mouse). Muscles, lungs, spleens, intestines, livers, and brains were derived from CBA mice at different times after induction, and 10% suspensions of organs in RPMI 1640 were used. For the destruction of cells, suspensions were twice frozen in liquid nitrogen, followed by thawing in warm water. After centrifugation at 1500*g*, IFN levels in supernatants were determined in L-929 murine cells using EMCV as a test virus.

TABLE 3. ANTIVIRAL EFFECTS OF POTENTIAL IFN INDUCERS

Substance	*Spectrum of action*
Amixin	Influenza, hepatitis A, rabies, viral encephalitis, herpetic lesion, cytomegalovirus (CMV), systemic lupus erythematosus[15,47]
Cycloferon, Neovir	Viral encephalitis, hepatitis B, rabies, HIV-1[15,56]
Megasin	Herpetic lesion[15]
Kagocel	Influenza, rhinoviral infections, rabies, viral encephalitis[15]
Ragosin	Hepatitis, enteroviral infections, rabies[15]
Savrats, Gosalidon	Hepatitis, herpetic lesion, chlamydiosis[15]
Poludan	Herpetic and adenoviral diseases of eyes[57]
Polyguacil	Influenza, hepatitis B, viral encephalitis, rabies, HIV-1[15,56]
Ampligen [poly(I)•poly(C_{12}U)]	HIV-1[15]
Ridostin	Various forms of herpes diseases, chlamydiosis, influenza, viral encephalitis, rabies, HIV-1[15,56,58–60]
Larifan	Various forms of herpes diseases, chlamydiosis, influenza, viral encephalitis, CMV, rabies[15,61–63]

are effective against experimental hepatitis[49,50] and against hepatitis A and other enteroviral infections in clinical trials.

IFN production in brain proceeds only after induction with low molecular weight compounds that are able to penetrate through the blood-brain barrier. Among these are Amixin, Cycloferon, and Kagocel. They are active against viral encephalitis.[51,52]

The method of application of the inducers strongly influences the induction of circulating IFN.[4,8,13,17,29,32,34,36] IFN circulating in the bloodstream in response to inducers have great significance in the tactics of inducer application. Circulating IFN represents the sum of IFN produced by different organs. The kinetics of IFN accumulation after induction differs with the inducer and may reveal the necessity for repeated injection of the inducer. If the level of circulating IFN reaches its maximum, additional inducer application is useless for at least several days. During this refractory period, repeated injection of the same inducer does not lead to additional IFN synthesis. It has been suggested that the refractory state prevents further use of IFN inducers as therapeutic remedies. When this period of hyporeactivity is over, however, the ability of the organism to synthesize IFN is gradually restored, and a few days later, it is possible to use the same inducer. The duration of the refractory state depends on the chemical structure of the inducer. Thus, elimination of the refractory state and effective use of the inducer require knowledge of how long the refractory period lasts.[53]

All known IFN inducers can be divided conditionally into two categories. The first includes compounds that stimulate production of the so-called early IFN. Among these are Larifan, which induces IFN formation that appears in the blood at 6–8 h after injection.[5,13,21,24] Ridostin stimulates two peaks of IFN production. The early peak appears in the serum at 4–6 h after induction; the other peak occurs at 24 h.[8,9,24] The refractory state that develops in response to both these representatives of dsRNA lasts for 3 days.[53] Cycloferon induces IFN concentrations that drop very rapidly about 2 h after induction. Twenty-four hours later, IFN disappears from the bloodstream almost completely.[4,24] The refractory state developed in response to this preparation continues for 2 days.

The other inducers stimulate production of late IFN. The first representative of this group, Amixin, induces IFN production that reaches its maximum at 18–24 h and disappears 48 h after induction.[4,17,24] Hyporeactivity in response to Amixin lasts for 4 days.[17]

The kinetics of IFN production induced by the derivatives of gossypol differs markedly from that stimulated by the other inducers. These compounds stimulate prolonged synthesis over several days of late IFN. For example, IFN induced by Kagocel reaches its maximum 48–72 h after induction.[24,29,32,34] Ragosin, on the other hand, induces two peaks of IFN in serum, one at 4 h, the other at 48–72 h.[24,36]

SPECTRUM OF ANTIVIRAL ACTIVITY BY IFN INDUCERS

The effectiveness of selected IFN inducers has been investigated against various viral infections, and it has been determined that the spectrum of activity for each preparation determines its further clinical application. The results revealed a most important property of these IFN inducers, an extraordinary broad range of action[6,14,15,54] (Table 3).

Low molecular weight compounds, such as Amixin and Cycloferon, have been found to be active against acute viral infections (influenza, hepatitis A),[47] as well as against chronic diseases (herpesvirus diseases, systemic lupus erythematosus, progressive systemic sclerosis).[55,64] On the other hand, nitric bases (Cycloferon, Neovir) that induce early IFN production are most effective against acute viral infections (encephalitis, hepatitis, rabies, HIV-1).[44,51,56]

Polymers are also effective under different circumstances. Poludan is given only locally in clinical practice because of its poor solubility. It is effective against herpetic and adenoviral diseases of the eye.[57] Polyguacil is effective against influenza, hepatitis B, viral encephalitis, rabies, and HIV-1. Several properties of natural dsRNA (Ridostin and Larifan) are especially effective against various forms of herpes diseases and chlamydiosis.[61–63,65] Both of these inducers are active against influenza, encephalitis, and rabies.[48,58–60] In addition, Ridostin activity might be applicable as a potential treatment for HIV-1.[56] Low molecular weight polyphenols are effective against influenza (Kagocel), herpes (Megasin), encephalitis and rabies (Savratz, Gosalidon), hepatitis, enteroviral infections, and HIV-1 (Ragosin).

Clinical trials of IFN inducers are limited at present to local application (herpetic diseases of the eye and skin, influenza, and rhinovirus infections)[47,57] and by the first attempts to apply them against chlamydiosis, hepatitis A, B, and C, and cytomegalovirus (CMV) infection. We hope that in the near future IFN inducers will be applicable against such conditions as encephalitis, rabies, systemic lupus erythematosus, progressive systemic sclerosis, and AIDS. Some of them, such as Amixin, Cycloferon, Ridostin, Larifan, and Poludan, are permitted for clinical application by the Pharmacological Committee of Public Health Ministry RF. Our data show that the antiviral activity of these specific preparations was not as effective as commercial preparations of IFN, although they had the same spectrum of activity. More detailed information about the structure and the mechanism of action of these preparations has been reported.[15]

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