РМЖ®

MEDICAL REVIEW

№ 18, 2011

SELECTED ARTICLES
FOR FAMILY DOCTORS

INDEPENDENT PUBLICATION FOR PRACTISING DOCTORS

www.rmj.ru

Increases in the efficacy of antiviral therapy and prophylaxis in acute respiratory viral infection. New experiments data from cell cultures

M. Yu. Eropkin, T. S. Bryazzhikova, and E. M. Eropkina

FGBU Science Research Institute of Influenza, Russian Federation Ministry of Health and Social Development, St. Petersburg.

Acute respiratory viral infections (ARVI) are among the most common diseases, accounting for up to 90% of all infectious disease. Influenza viruses A and B have the greatest epidemic significance. Nonetheless, pathogens belonging to different families with different morphologies and genetic characteristics but producing clinically similar diseases make tangible contributions to the overall pattern of respiratory infections, with stable levels of incidence during the seasons between influenza epidemics. The most common aetiological agents are parainfluenza virus, respiratory syncytial virus, and adeno- and coronaviruses. Retrospective analysis and contemporary data show that this tendency in general terms persists in different years and different territories (Fig. 1) [1-3].

In the structure of acute infections of the respiratory tract, parainfluenza viruses account for about 20% in the adult population and 30-40% among young children, second only to respiratory syncytial virus as the aetiological factor in infections of the lower respiratory tract [1]. Parainfluenza viruses are RNA-containing paramyxoviruses, of size 100-300 nm. A total of 4 types of parainfluenza virus isolated from humans are now known. Unlike influenza viruses, they are not characterised by variability in their antigenic structure. In most patients, parainfluenza occurs as a transient illness (lasting no more than 3-6 days) without severe general intoxication. However, the disease may be apparent as croup and variants of bronchiolitis and pneumonia. Parainfluenza types 1 and 2 are most commonly associated with croup, while the most pathogenic parainfluenza virus, type 3, causes bronchiolitis and pneumonia more often than the other types. Children in the first year of life are particularly susceptible to parainfluenza. Thus, the role of parainfluenza infection in mortality among young children and immunosuppressed adult patients must be noted; complicated by bacterial infections, these viruses can be the cause of death from lower respiratory tract infections in 25-30% of cases in these groups. Reinfection with parainfluenza can occur throughout life and is of particular importance in immunosuppressed patients, of which there are increasing numbers because of unfavourable ecological conditions and stressful city life.

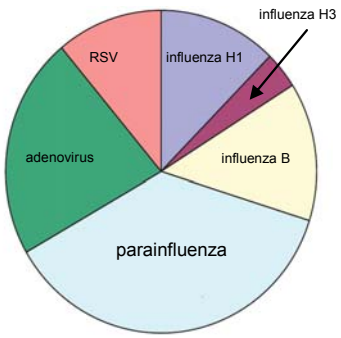

Fig. 1. Aetiological distribution of ARVI in Russia in the 2010-2011 epidemic season, data from the Federal Influenza Centre (Science Research Institute of Influenza) [2]. All types of influenza - 29.1%; all ARVI other than influenza - 70.1%.

Respiratory syncytial virus, also a member of the RNA-containing paramyxovirus group, is an important pathogen in lower respiratory tract infections in the young. From 80% to 90% of all youngsters experience at least one episode of RSV infection, 0.5-2% requiring hospital treatment [4]. These authors found that 30% of adults who had had RSV infections in childhood developed bronchial asthma, as compared with 3.8% in the control group.

Adenoviruses and coronaviruses, which are indispensable participants in the epidemic process, account for up to 50% and up to 20% respectively in the structure of ARVI [5]. Adenoviruses are non-enveloped DNA-containing viruses up to 80 nm in diameter, with more than 50 serotypes differing in terms of their epidemiological characteristics and

tropicities for different tissues. Thus, the spectrum of diseases they cause is quite wide: inflammatory diseases of the nasopharynx, conjunctivae, urinary bladder, and gastrointestinal tract. Adenoviruses are isolated from up to 7.5% of cases of acute intestinal infections [6]. We focused on adenovirus serotype 3 because of its great epidemiological significance as a pathogen of nasopharyngeal fever. As regards coronaviruses, which are RNA-containing viruses whose virions have a lipoprotein envelope and a size of about 120 nm, these, like adenoviruses, are tropic not only for the respiratory tract epithelium but also for the epithelium of the gastrointestinal tract. Young children are particularly susceptible to coronavirus infection, where catarrhal signs are in a vast majority of cases combined with diarrhoea [5].

Another point regarding respiratory viruses which should be noted is that antiviral therapy is of value in high-risk groups and in patients with severe illness. Thus, ribavirin is prescribed to patients with risk factors and severe RSV infections [1]. In other cases, treatment of acute respiratory virus infections is directed mainly at the major component of pathogenesis. The search for substances easing the course of illness and reducing its duration with a minimum of side effects is a relevant task. No less relevant than the question of using aetiotropic agents is the rapid appearance and selection of resistant strains. Thus, remantadine, not only being ineffective against influenza B, has been ineffective in recent epidemic seasons against some viruses of the A(H3N2) subtype. Seasonal viruses of the A(H1N1) subtype, conversely, were mostly sensitive to remantadine but rapidly acquired resistance to oseltamivir [7]. The 2009 pandemic strain A(H1N1)pdm is sensitive to oseltamivir and resistant to remantadine [8]. These points provide grounds for seeking agents not directed at a virus per se or its interaction with cells, but stimulating cellular resistance, interferon production, and immune protection. Thus, the use of ***non-specific anti-ARVI agents*** remains relevant, these including plant and homoeopathic preparations.

Influcid (*Deutsche Homöopathie-Union*) is one such agent and has the following composition: 100 g of solution contains Aconitum D3 10 g, Gelsemium D3 10 g, Ipecacuanha D3 10 g, Phosphorus D5 10 g, Bryonia D2 10 g, and Eupatorium perfoliatum D1 10 g. Other ingredients are: Eucalyptus globules, 96% ethanol, and purified water. The alcohol content is 45% v/v. The preparation is released in liquid and tablet forms. Published data show that Influcid significantly decreases symptoms common to all acute respiratory infections, such as hyperthermia, limb pain, cough, mucosal hyperaemia, and inflammation in the pharynx and larynx [9]. *In vitro* studies have demonstrated a stimulating action on cellular interferon production [10].

We have previously demonstrated the antiviral action of Influcid *in vitro* against a whole series of current strains of influenza - seasonal A(H1N1) strains and the 2009 pandemic strains A(H1N1)pdm, A(H3N2), A(H5N1), and B, as well as human herpesviruses types 1 and 2 and adenovirus type III [11-13]. The agent not only decreased the production of virus particles when assayed by the haemagglutinin reaction (HA), but also increased the resistance of cultured cells to the cytopathogenic actions of viruses when assessed in terms of cell viability and respiratory metabolism using the MTT test [12, 13]. Furthermore, significant reductions in mortality in mice infected with lethal doses of influenza A/PR/8/34 were demonstrated using therapeutic-prophylactic clinically adequate doses of Influcid [11].

The aim of the present work was to perform a comparative *in vitro* study of the actions of Influcid against standard parainfluenza, coronavirus, and RSV strains and its potential cytoprotective action in relation to the toxicity induced by high concentrations of the widely used antiviral agents remantadine and arbidol. Standard virus strains from the Upper Respiratory Infection Virus Collection, Science Research Institute of Influenza, Ministry of Health and Social Development were used: HPIV-3/2235, HCoV-lp/3482, and RSV/4317, as well as HSV1/SPb/248/88 and HSC2/etalon/2000. Cell cultures were A-549 cells (an epithelioid line derived from a human lung carcinoma) and L-41 cells (a human macrophage line of monocyte-leukaemic origin).

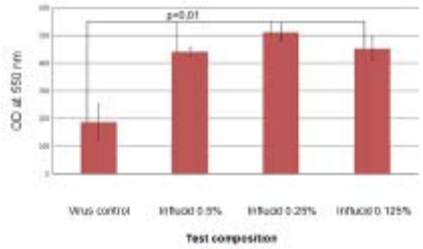

Fig. 2. Antiviral action of Influcid on type 3 adenovirus in L-41 cells. The viral load was 10TID$_{50}$.

Virus cytopathic effects were assessed under an inverted microscope. The second important indicator of the protective effects of the agent consisted of the ability of cells in culture to reduce the tetrazolium stain MTT (thiazolyl blue), the intensity of which reflects the level of cell viability in terms of the ability of their mitochondria and, partly, cytoplasmic dehydrogenases, to reduce the stain. This test is widely used in virology for assessing the cytopathogenic effects of viruses on cells [14]. The results can be interpreted as the level of cellular resistance to virus effects. The microtetrazolium test is also widely used to assess the effects of toxic substances, pharmacological agents, and unfavourable environmental factors on cells [15], so it allows simultaneous *in vitro* assessment of antiviral effects and the toxicity of study agents. Influcid was added to the culture medium 6-24 h before virus infection ("prophylactic" scheme) or simultaneously with virus infection ("therapeutic-prophylactic" scheme) in PBS at concentrations ranging from 0.125% to 0.5% (the concentration in the medium used for the initial liquid formulation).

Results

The antiviral effects of different Influcid concentrations against type III adenovirus at a virus load of 10 tissue infective doses are shown in Fig. 2. The criterion for cultured cell viability was optical density in the MTT test. As shown in Fig. 2, there was an optimum concentration at which the agent was most effective. The lowest effective concentration, 0.125%, was selected for further studies.

The antiviral effects of Influcid against the ARVI viruses studied in the therapeutic-

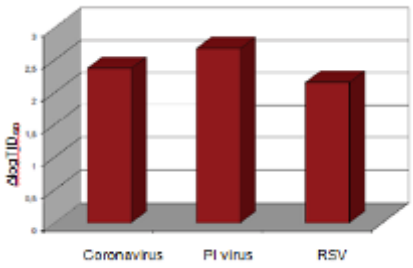

Fig. 3. Effects of Influcid (0.125%) against ARVI viruses on simultaneous addition of Influcid and virus to the culture medium ("therapeutic-prophylactic" scheme).

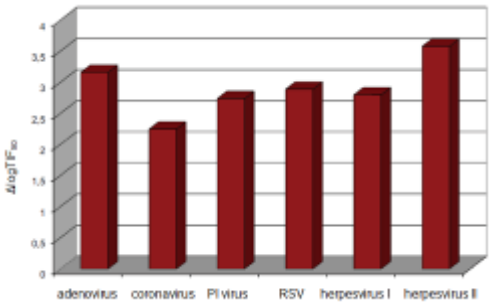

Fig. 4. Effects of Influcid (0.125%) against ARVI viruses and herpes on addition to cultures 6 h before cell infection ("prophylactic" scheme).

prophylactic scheme are shown in Fig. 3. The efficacy criterion was the log difference in virus titres between controls (in the absence of agent) and experiments, i.e., $logTID_{50}$. Generally in virology studies, $logTID_{50} \geq 2.0$ is regarded as a convincing antiviral effect. The results show that this agent was effective against all the viruses studied.

As expected, preincubation of cell cultures with Influcid followed by infection with viruses yielded even more marked protective effects (Fig. 4). Differences in virus titres from controls were greater than 2.5, even reaching 3, for most of the viruses tested, which for a wide-spectrum agent is a very good result. There are good reasons for presenting data on herpesviruses along with ARVI viruses in Fig. 4. In the light of the previously demonstrated stimulatory action of this agent in relation to cellular interferon production [10], there is great interest in the high efficacy of Influcid against type II herpesvirus, as infection with these viruses is associated with immunodeficiency states: disease due to herpes simplex virus is seen clinically as infections complicating immunodeficiency states of various aetiologies as well as a disease in its own right, which is regarded as secondary immunodeficiency. Many authors have shown that type II herpesviruses induce significantly more severe and diverse immunological impairments than type I, resulting in frequent recurrences, often resistant to aetiotropic therapy [16].

The next part of our study addressed the possible protective effect of Influcid in relation to the toxic responses of cultured cells to high doses of the antiviral agents remantadine and arbidol. The basis for these

Fig. 5. Protective effects of Influcid (0.125%) against toxicity induced by high remantadine concentrations in L-41 cell cultures. MTT test, photograph of plates.

experiments was that the antiviral actions of Influcid *in vitro*: 1) are non-specific; 2) have a cytoprotective action as the main mechanism, i.e., the agent protects cells against the cytopathic effects of viruses. Fig. 5 shows 96-well plates (L-41 and A-549 cultures) exposed to decreasing concentrations of remantadine with concentration ratios of 1:1.4 (upper 4 rows) and the same concentrations of remantadine in the presence of Influcid

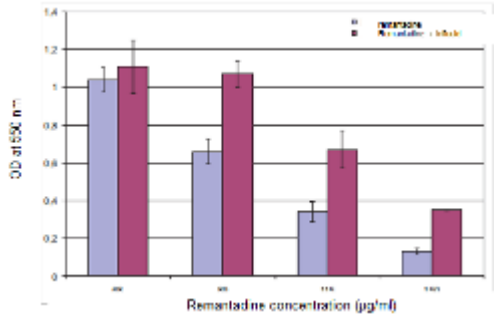

Fig. 6. Protective effects of Influcid (0.125%) against toxicity induced by high concentrations of remantadine in L-41 cell cultures; photometry data.

Fig. 7. Protective effects of Influcid (0.125%) against toxicity induced by high arbidol doses in A-549 cell cultures. MTT test, photograph of plate.

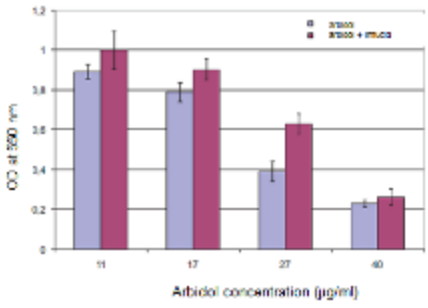

Fig. 8. Protective effects of Influcid (0.125%) against toxicity induced by high arbidol doses in A-549 cell cultures. MTT test, photometry data.

(0.125%). Influcid was added as per the "prophylactic" scheme. Staining was by the MTT method. Figure 5 shows that Influcid had protective effects at the borderline remantadine concentrations, which were toxic but did not kill all the cells. This was supported by quantitative data obtained by spectrophotometric measurement of the plates on a Varioscan analyser at the characteristic wavelength of 550 nm (Fig. 6) - Influcid had significant protective effects at virtually all toxic remantadine concentrations.

Similarly, Influcid had *in vitro* activity against the toxicity of the contemporary anti-influenza agent arbidol (Figs. 7 and 8). It should be emphasised that both remantadine and arbidol were used in this study at concentrations known to be higher than those corresponding to therapeutic doses. However, an important consequence of the effect seen was the possibility of using Influcid in combination with these agents in ARVI, with increases in doses without the risk of developing side effects.

Conclusions

1. Influcid has *in vitro* antiviral activity against a wide spectrum of viruses causing ARVI: adenoviruses, coronaviruses, respiratory syncytial virus, and parainfluenza viruses. The agent was particularly effective when used in the prophylactic regime, i.e., given 6-24 h before cell infection.
2. In view of the previously demonstrated interferon-stimulating action of Influcid, the high *in vitro* efficacy of this agent against herpes simplex virus, especially type 2, which is linked with the development of secondary immunodeficiencies, is of great interest.
3. Apart from its antiviral effect, Influcid had general cytoprotective actions, protecting cultured cells from the toxic actions of high concentrations of the antiviral agents remantadine and arbidol.

References

1. Subhash Chandra Parija http://emedicine.medscape.com/article/224708–overview/Updated:Apr 19, 2010.
2. www.influenza.spb.ru
3. Голованова А.К., Ревунова О.В., Цыбалова Л.М. и др. Этиологическая структура респираторных вирусов в Санкт–Петербурге и Ленинградской области по результатам их изоляции в 1997–2000 г.г. // В сб.: Современные аспекты вакцинопрофилактики, химиотерапии, эпидемиологии, диагностики гриппа и других вирусных инфекций. СПб., апрель 2001, С.16–18.
4. Ruotsalainen M. et al. Respiratory morbidity in adulthood after respiratory syncytial virus hospitalization in infancy. // Pediatr. Infect Dis. J. – 2010. – V. 29(9). – P. 872–974.
5. Смирнов В.С. Современные средства профилактики и лечения гриппа и ОРВИ. –СПб., «ФАРМиндекс» – 2003. – 50С.
6. Козина Г.А. Клинико–эпидемиологические особенности и вопросы терапии острых кишечных инфекций аденовирусной этиологии F 40/41 у детей. / Автореф. на соиск. уч. ст. канд. мед. наук. М., 2010г.
7. Еропкин М.Ю., Грудинин М.П., Коновалова Н.И. и др. Антигенные и генетические особенности современных вирусов гриппа в России // Материалы I Всероссийского Ежегодного Конгресса по инфекционным болезням. М., 30 марта–01 апреля, 2009. – С. 67.
8. Киселев О.И., Комиссаров А.Б., Стукова М.А. и др. Пандемический грипп 2009 г. в России. Диагностика и молекулярно–биологические характеристики вируса // Вопр. вирусологии. – 2011. – Т. 56. – № 1. – С. 17–21.
9. Гаращенко Т.И., Гаращенко М.В., Мезенцева М.В. Клинико–иммунологическое обоснование гомеопатических препаратов в профилактике и лечении гриппа и ОРВИ // Русский медицинский журнал.–2005.–Т.13.–№13.–С.1432–1437.
10. Ершов Ф.И. Рациональная фармакотерапия гриппа и ОРВИ // Фарм. вестник. – 2003. – № 33(312).
11. Еропкин М.Ю., Григорьева В.А., Гудкова Т.М. и др. Активность препарата «Инфлюцид» в отношении вирусов гриппа в модельных системах // МЕДЛАЙН–Экспресс. – 2007. – № 6. – С.23–26.
12. Еропкин М.Ю., Коновалова Н.И., Григорьева В.А., Байбус Д.М., Гудкова Т.М. Действие препарата Инфлюцид in vitro против пандемического штамма 2009 г. A(H1N1) «свиного» («мексиканского») гриппа // Традиционная медицина – 2009. – № 3(18). – С. 14–18.
13. Еропкин М.Ю., Гудкова Т.М., Даниленко Д.М. и др. Пандемический грипп 2009 г. в России: антигенные, биологические свойства и чувствительность к противовирусным препаратам // Русский медицинский ж. – 2010. – Т. 18, № 7. – С.410–415.
14. Watanabe W., Konno K., Ijichi K., et al. MTT colorimetric assay system for the screening of anti–orthomyxo– and anti–paramyxoviral agents // J. Virol. Methods. 1994. Vol. 48 No.2–3 P. 257–265.
15. Borenfreund E, Babich H, Martin–Alguacil N. Comparison of two in vitro cytotoxicity assays – the neutral red (NR) and tetrazolium (MTT) tests // Toxicol. In Vitro. – 1988. Vol. 2 No. 1. P. 1–6.
16. Брязжикова Т.С. Герпетическая инфекция: профилактика и лечение // Врач. – 2004. – №2. – С.47–48.