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Review

Dipyridamole as an Interferon Inducer

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Abstract

We described the pyrimidine derivative dipyridamole (DP) as an interferon (IFN) inducer in cultivated *in vitro* lymphoid cells, administrated orally in mice and in humans attaining high IFN titres within 24 hours. Gut-associated lymphoid tissue was identified as the inducer target. IFN-antagonistic period was established. Three double-blind placebo trials (more than 6000 participants proved that DP acts as a prophylactic for influenza and viral acute respiratory diseases. Experimental evidence proved that the IFN-inducing activity of DP is due to its capacity to inhibit cAMP phosphodiesterase. Additionally, DP demonstrates a marked inhibitory effect on the replication of a broad spectrum of viruses. Iт was established that DP-induced IFN production in humans is strongly dependent of compound pharmacokinetics.

DP activities were followed and described in lupus erythematosus, chronic pulmonary diseases, liver diseases, in ophthalmology, dermatology (herpesvirus and papillomavirus infections) and AIDS. Special attention was given to a possible DP's effect in COVID-19 patients.

Keywords: dipyridamole, interferon, double-blind trials, acute respiratory infections

Резюме

В серия публикации описахме пиримидиновото производно дипиридамол (DP) като индуктор на интерферон (IFN) в култивирани *ин витро* лимфоидни клетки, при перорално прилагане на мишки и у хора, достигайки високи титри IFN в течение на 24 часа. Чревната лимфоидна тъкан бе посочена като мишена на този ефект на DP. Регистриран бе период на антагонизъм към индуктора. Проведените три изпитания по схемата double-blind placebo-controlled trials (върху над 6000 души) доказаха, че DP може да се прилага за профилактика на епидемичния грип и на вирусните остри респираторни инфекции. Експерименатално бе установено, че IFN-индуциращата активност на DP се дължи на способността му да инхибира фосфодиестеразата на cAMP. Освен това, DP показва отчетлив инхибиращ ефект върху репликацията на широк спектър вируси, принадлежащи към различни таксономични групи. Доказано бе, че DP-индуцираната продукция на IFN у хора е строго зависима от фармакокинетиката на съединението. Активност на DP е описана и проследена при lupus erythematosus, хронични заболявания на белите дробове, чернодробни заболявания, при очни заболявания, в дерматологията (херпесни и папиломавирусни инфекции) и при СПИН. Специално внимание е отделено на възможния ефект на DP при пациенти с COVID-19.

Interferon-inducing activity of dipyridamole

In 1982 we described and characterized 2,6-bis(diethylamine)-4,8-dipiperidino-pyrimido-[5,4-d]-pyrimidine (dipyridamole, DP, Persantin©, Curantyl©, Antistenocardin©), a compound extensively used as an antiaggregant and vasodilator, as an IFN inducer (Galabov and Mastikova, 1982a). The ability to induce IFN was manifested both *in vitro* - in lymphoid cell cultures (exogenous IFN) – and *in vivo* - in experimental animals (mice, turkeys, hens) (endogenous IFN) (Galabov and Mastikova, 1983; Protasova, 1996). DP-induced IFN was established in explanted *in vitro* mouse peritoneal leucocytes and in Namalva lymphoblastoid cells (Galabov and Mastikova, 1982a), cultured human leucocytes (Grigorian *et al*., 1989), tonsil lymphoid cells

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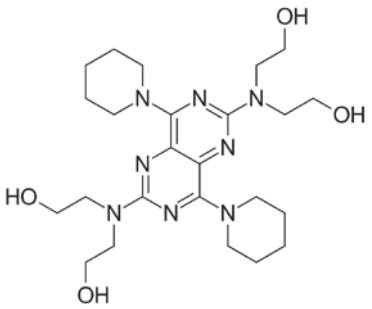

and blood leucocytes and mouse bone marrow cells. Oral administration in mice resulted in substantially high levels of IFN both in sera and organs (4100–8200 IU/mL in sera), which persisted until Day 7 (Galabov and Mastikova, 1983; Protassova, 1996). A single oral administration of DP at an optimal IFN-inducing dose demonstrated a marked antiviral effect in mice infected with influenza viruses A (H1N1) and B, Semliki Forest virus and herpes simplex virus type 1 (Mastikova, 1985; Mastikova *et al.*, 2019). Experimental evidence showed that *in vivo* (in mice), DP induces IFN in gout-associated lymphoid tissue. DP induction of IFN was also established in hens (Manafova *et al*., 1985). Some IFN-inducing capacity was also recorded in non-lymphoid cell cultures.

Oral administration of DP (25 mg/kg) in mice manifested high IFN titres in spleen at 12-24 h (≥ 2048 IU/mL) and in liver at 24 h (256 IU/mL). Increased levels were also established in kidneys (128 IU/mL), lungs (256 IU/mL) and brain (64 IU/mL). Using the test of neutralization of IFN activity *via* mouse anti-IFN serum showed that the DP-induced substance is an IFN of the first type; it is relatively labile (partially inactivated at 56ºC within 60 min and at 65ºC for 30 min) and is stable within 30 min at 56ºC (Mastikova, 1985). DP, as an IFN inducer, also demonstrated immunomodulatory properties (Koychev *et al*., 1985; Michaylova *et al.,* 1985).

Because the pharmacological and toxicological properties of DP were well studied in thorough trials, especially clinical trials, it was possible to move on to investigating DP's IFN-inducing properties in humans. It was established that a single oral intake of 100 mg (1.0-1.5 mg/kg) DP induced a marked increase of IFN in 90% of the adult volunteers tested at 24-48 h, with an individual variation from 32 to 8200 IU/mL (average 958 IU/mL). In addition, 87.5% of the serum IFN produced was HuIFN-α (Galabov and Mastikova, 1984a, b). In both mice and humans with an IFN response to DP, the phenomenon of hyporeactiveness to a consecutive IFN induction was explored. It was found that the period of hyporeactiveness lasted from Day 4 to Day 6 in humans (Galabov and Mastikova, 1984a, b). These data laid the groundwork for developing an application scheme for this IFN inducer as a means of prophylaxis and treatment of viral infections. In fact, DP was the first IFN inducer whose pharmacological and toxicological characteristics permitted an epidemiological trial.

Thus, three double blind trials were organized and carried out in Bulgaria to demonstrate the efficacy of DP as a means for prophylaxis of influenza and viral acute respiratory diseases (Kuzmov *et al.*, 1985; Kozhukharova *et al*., 1987). These trials took place during the seasonal epidemic wave of influenza, in administrative and industrial enterprises in Sofia (in the first trimester of 1983) and in Dobrich (1985) and involved adult participants (over 19 years old). The number of individuals necessary for the trials was determined according to Bessmertnij and Heyfets (1963). Drug (DP) and placebo groups were formed according to requirements for identical working conditions and for achieving maximal density and involvement for both groups. In total, 8 249 people were included in the trials: 4 343 on DP and 3 906 on placebo. DP - a product of the Chemio-Pharmaceutical Work of Sofia (Pharmachim©) - was provided as dragees of 25 mg, along with identically looking placebo dragees. They were administered according to the following schedule: 100 mg (4 dragees taken in pairs at 2-h intervals) once each 7 days throughout the entire epidemic period (January-March). Evaluation of DP's prophylactic efficacy was carried out by comparing the wholesale incidence of influenza and acute respiratory diseases in DP and placebo groups. Data were taken from the official registrations of the diseases. People who had received less than two-thirds of the drug or placebo applications planned were excluded from the analysis. Evaluation had to meet the following criteria: (1) the significance level of the difference in morbidity in drug and control groups - p (chi^2), (2) the index of epidemiological effectiveness (IE), its scope of confidence, (3) the subsequent protection index (PI). In addition, the following tests were included in the trials: (a) retention of haemagglutination reaction (RHR) for influenza and parainfluenza viruses, (b) complement fixing reaction for adenoviruses, respiratory syncytial viruses and mycoplasma pneumoniae with a wide range of antigens on paired serum samples obtained before onset and after closure of the trial period, (c) determination of the IFN titres. Results of the trials are shown in Tables 1 and 2.

Table 1. Epidemiological efficiency of DP applied for prophylaxis of influenza and other viral acute respiratory diseases (VARD)

Group	Number of people under survey	Diseased (VARD)		Epidemiological efficiency index
		Number	%	
Trial 1				
DP	749	72	9.6	1.7÷2.1÷3.1
Placebo	501	104	20.7	
Trial 2				
DP	328	64	19.5	1.3÷1.9÷2.7
Placebo	303	113	37.2	
Trial 3				
DP	2725	178	6.5	1.2÷1.6÷2.1
Placebo	2664	283	10.6	

Table 2. DP effects on seropositivity and IFN production in people included in Trial 1

Group	Seropositive vs VARD		*p*	IFN blood level 4-fold increase		*p*
	N/total	%		N/total	%	
DP	18/40	45.0±15.4	0.05	10/33	30.3 (16.1÷46.7)	0.05
Placebo	28/41	68.3±14.2	0.05	1/35	2.8 (0.01÷10.8)	0.05

Analysis of the results of all three trials showed a markedly decreased incidence of influenza and viral acute respiratory diseases in the groups on DP. The statistically significant differences between DP and placebo groups support the thesis of a protective effect of DP (Kuzmov *et al.,* 1985; Kozhukharova *et al.,* 1987). Subsequent investigations carried out in industrial enterprises in Russia (in Moscow) during flu epidemics and seasonal waves of other acute respiratory viral infections demonstrated the marked preventive effect of DP. In sum 4 559 persons were embraced in these studies (2 234 receiving DP and the rest - placebo control group). The disease incidence was decreased by 2.66 times, in the risk groups - by approximately 5 times (effectivity index of 4.81) (Slepushkin and Fedorova, 2000; Kareva, 2015).

Data in the literature concerning the biochemical properties of a number of low-molecular-weight IFN inducers, to which DP should be added, shows that some of these IFN inducers are also inhibitors of the phosphodiesterase (PDE) of cAMP. These include tilorone hydrochloride and other fluorenone derivatives, acridine derivatives (10-carboxymethyl-9-acridanone (CMA) being especially potent), aryl pyrimidinones, and so on. Hait and Weiss (1979), working on splenocyte homogenates, proved that DP is a cAMP PDE inhibitor. We confirmed these data and determined the quantitative parameters of the process of cAMP PDE inhibition by DP. We carried out a kinetical analysis of DP inhibitory action on cAMP PDE from mouse peritoneal leukocytes (MPL) and from mouse NIH 3T3 cells, which are known as a standard source of this enzyme. We used Lineweaver and Burk's (1934) method to prove that DP is a competitive inhibitor of cAMP PDE. In addition, we used Dixon's (1953) method for determining the value of the inhibitor constant (Ki_{med}) of DP: 4.6×10^{-5} M (Galabov *et al.,* 1985).

Mechanism of IFN induction by dipyridamole

A series of publications demonstrated both the IFN-inducing and regulatory functions of DP, discussed by Surkina (2000). Hait and Weiss (1979) initially demonstrated, *via* experiments in mouse splenocyte homogenates, that DP is an inhibitor of cAMP. This was confirmed in our investigations using homogenates of other cell species - mouse peritoneal leucocytes and NIH 3T3 cells. In addition, a series of data presented the quantitative characteristics of DP-inactivated cAMP PDE. It was found that DP is a competitive inhibitor of this enzyme, and the inhibition constant value (K_i) was determined. It was established that DP's K_i is 10-fold higher than that of the standard cAMP PDE inhibitor theophylline (Itkes and Kochetkova, 1981). As the NIH 3T3 cell lysate contains a single kinetic form of this enzyme, the results characterized the interaction of DP with that individual enzyme and not with an isoenzyme mixture (Galabov *et al*., 1984).

The influence of DP on cAMP synthesis in mouse peritoneal cells was studied following Itkes *et al.* (1982). Two peaks of increased cAMP levels were observed, the first at 2 h (6.4 x 10^4 IU/mL) and the second within the 8-22 h period. The first peak was DP dependent, and the second was IFN dependent. The DP-dependent increase of cAMP levels preceded the production phase - for example, it coincided with the induction phase (Galabov *et al*., 1985). These data are consistent with the hypothesis of the direct participation of cAMP in DP-induced IFN production. This concept is consistent with data in the literature showing that among low-molecular weight IFN inducers, the strongest are without exception cAMP PDE inhibitors. Analysis of all data on the effects of tilorone and other fluorenone derivatives, acridine derivatives (including 10-carboxymethyl-9-acridinone), aryl pyrimidinones and others shows that all these substances are inhibitors of cAMP PDE.

Dipyridamole's effect and neoplasms

In connection with Inglot and colleague's publications (Havlin *et al*., 1988; Groehlich *et al*., 1988), investigations into the IFN response in animals with virus-induced neoplasms has to be mentioned. In mice with markedly developed lymphoma, a very low IFN level (16 IU/mL at maximum) was established in sera after oral administration of DP, in contrast to the very high titres in healthy animals (> 2000 IU/mL). DP applied orally at a dose of 25 mg/kg and 100 mg/kg did not induce IFN production in female mice (20 g body weight) with Rauscher lymphoma (Yakimov *et al*., unpublished data, 1984). In these experiments, the serum IFN was assayed *in vitro* in cultivated mouse peritoneal cells.

DP also does not induce marked IFN levels in chickens (average 40 g) or turkeys inoculated with E-26 erythroblastosis virus (T. L. Galabova *et al*., unpublished data, 1984). These data partially explain Havlin *et al.*'s (1988) finding of the lack of IFN production by DP in 43 humans, following oral administration of 150 mg in a single intake or 887 mg intravenously. The great majority of people included in this study, organized by the Polish researcher Anna Inglot (Wroclaw), were patients with neoplasms at different stages of cancer development. It was found that DP enhances the antiproliferative effect of IFN in various human tumour cells (Suzuki *et al*., 2008).

Pharmacokinetics-dependence of dipyridamole-induced IFN synthesis in humans

In parallel with the Havlin *et al.* (1988) study, the Polish co-authors in that study (Groehlich *et al*., 1988) published their own results showing that DP did not induce IFN in 34 healthy adult volunteers, 22 of whom received the compound orally at a single dose of 150 mg, while the rest received it intravenously (10 mg). The IFN assay was done on the human lung carcinoma cell line A549. There is reason to consider the dose dependence of the DP-induced IFN production (Gurevich and Surkina, 2001): double administration of the drug in a total dose of 100 mg shows the IFN-induction effect (Galabov and Mastikova, 1984; Slepushkin and Fedorova, 2000), whereas no such action was observed when DP was taken in a single dose of 100 or 150 mg (Havlin *et al*., 1988; Tonew *et al.,* 1990).

Establishing a pharmacokinetic model of the antiviral action of DP administered orally in humans resolved the contradiction. A twofold 50-mg dosage ensures the optimum IFN biosynthesis by providing for a nearly maximum cAMP accumulation while being nontoxic; any further increase in the dosage may result in toxicity development (Gurevich and Surkina, 2001). A single administration of DP in a dose of 200-250 mg was reported to affect platelet aggregation and blood coagulation behaviour (Lukyanov and Belousov, 1998). The studied pharmacokinetic model describing the dose-effect relationship for the antiviral activity of DP explained the features of the DP-induced IFN synthesis observed with the twofold (2 x 50 mg) peroral administration of the drug and the absence of such features in the case of a single-dose (100 mg) administration of the same drug. Single administration of higher doses (as was done by Havlin *et al*., 1988) showed a markedly stronger effect - suppression of IFN synthesis.

In addition to the *in vitro* IFN assay used in these studies - the protocol of IFN titre determination and the cell culture used could be of critical importance and would help explain the contradiction in determining IFN productions. The methodology of IFN assay in our laboratory was described in Galabov and Mastikova (1982b).

Dipyridamole effect in lupus erythematosus

IFN levels were measured in serum samples from 22 patients with systemic lupus erythematosus (SLE) and 12 patients with discoid lupus erythematosus (DLE) before and 24 h after DP administration. Initial serum concentrations of α-IFN in SLE patients were markedly elevated. The percentage of DLE positive responders to DP induction was about twice as high as that found for SLE. IFN titres obtained on human diploid lung fibroblasts were almost identical to those on primary calf kidney cells,

which were used in a parallel IFN assay (Konstantinov *et al.,* 1989). DP has been used as adjunctive therapy for DLE (Marcus *et al.,* 2019).

Dipyridamole in common cold and chronic pulmonary diseases

After the initial data on DP's inhibitory effect on rhino virus replication *in vitro* (Tonew *et al.*, 1978, Oehring and Schmidt, 1978), it was found that DP demonstrated a marked protective efficacy against natural colds, attaining 49-62% (Jefferson *et al*., 2004). Investigations were carried out on 35 patients, aged 50-70 years, with chronic brochettes with pneumosclerosis, pleuritides, cor pulmonale, silicosis, and the like. Only 60% reacted with increased IFN levels (vs. 80-90% of healthy volunteers). Mean geometrical titres showed only a 30-fold increase of IFN levels in positively reacting patients (vs. a 130-to 200=fold increase in healthy volunteers) (Galabov *et al*., 1984b, 1987).

Dipyridamole in hepatology

It was found that DP applied orally in 42 patients with chronic hepatitis B infection did not result in IFN production; in contrast, a lack of IFN response was found in only 10% of the 40 healthy participants. The IFN response was related to the extent of liver lesions in the group of HbsAg carriers. The number of patients with positive IFN response was significantly smaller in the group of active hepatitis and cirrhotic patients compared with the group of healthy HbsAg carriers (Maleev *et al.,* 1985). It was established that DP suppresses hepatoma 3924A cells (Zhen *et al.*, 1983).

Dipyridamole in ophthalmology

DP was used orally in the treatment of patients with epidemic keratoconjunctivitis. The treatment course on 102 patients (out of 120 in total) was based on data in the literature for markedly elevated blood levels of produced IFN and for the hyporeactivity period in DP-induced IFN synthesis (4–6 days in humans). DP was applied at a dose of 100 mg, divided into two intakes within two hours on two consecutive days and then one dose per week. The widely accepted symptomatic means were added in the treatment course (sulphamide drops locally, resorcinol compress, vitamin C and sandocten calcium). In all patients (except 5 in which the start of DP treatment was delayed by a week), a marked improvement was observed. In the keratitis stage, only a diffuse keratitis without subepithelial infiltrates was seen. Moreover, no iridocyclitis was registered at all (Vassileva and Galabov, 1985).

Dipyridamole effects in dermatology

As mentioned above, a therapeutic effect of DP in patients with herpes simplex was found (Günther *et al*., 1977). The compound blocked the reactivation of thymidine kinase negative HSV (Tenser *et al*., 2001). In addition, DP potentiated the activity of various acyclic nucleoside phosphonates (antiviral agents with a broad spectrum of activity against retroviruses and DNA viruses) against herpes simplex virus, varicella-zoster virus and human cytomegalovirus (Snoeck *et al*., 1994). DP used in IFN-inducing doses in trials versus placebo prevented the recurrences of condylomata acuminata after diathermocoagulation (Mancuso *et al*., 1995).

Dipyridamole in AIDS

DP and 3'-azido-3'-deoxythymidine and other dideoxynucleosides act as synergists in AIDS therapy (Szebeni *et al.,* 1989; Weinstein *et al*., 1990, Arts and Hazuda, 2012).

Dipyridamole in COVID-19

DP is an appropriate antiplatelet drug. It has been found that a series of compounds are inhibitors of the platelet cAMP PDE (Meanwell and Seiler, 1990). DP is one of the strong inhibitors (Paolo *et al*., 2011). DP possesses selective anti-inflammatory properties, thus contributing to the secondary prevention of stroke. It inhibits inflammatory gene expression in platelet-monocyte aggregates (Weyrich *et al.,* 2005).

This effect of DP is the main reason for this drug to be included in the combination therapy of the SARS-CoV-2 infection (COVID-19) (Liu *et al*., 2020*;* Rogosnitzky *et al.,* 2020). Other effects, such as antiviral, anti-inflammatory and antioxidant effects, have also been taken into account in the anti-COVID-19 field. DP's antiviral effect in that connection was especially highlighted. Clinical data has underlined that DP may substantially improve the clinical outcomes of COVID-19 treatment. The pharmacokinetics profile of the drug is well established, which makes it easier to consider it an appropriate therapeutic course. DP is known as being generally safe, affordable and available worldwide. Despite the existing evidence showing its substantial promise in treating critically ill COVID-19 patients, however, larger randomized and controlled trials (double-blind trials) are needed to confirm its potential (Aliter and Al-Horani, 2020). In fact, DP (oral dose of 100 mg tablet four times a day) is currently in phase 2 of a double-blind trial on 80 volunteers, sponsored by the University of Michigan (Knight, 2020). The results obtained per-

mit to take in mind the possible effects of DP, not anti-aggregant only, in the treatment of COVID-19 patients.

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