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MOLNUPIRAVIR AS A PROMISING PRODRUG AGENT FOR THERAPY OF COVID-19

Abstract. The ongoing COVID-19 pandemic accompanied by the emergence of new successive pathogenic variants makes problematic the prospects of the approach based on application of exclusively prophylactic vaccines to combat SARS-CoV-2. This reason motivated the urgent need in search and development of chemical formulas showing direct antiviral action.

The present mini-review provides data on chemical and enzymatic methods of producing molnupiravir regarded so far as one of the most effective pharmaceuticals for treatment of COVID-19. In conclusion of the literature survey it is suggested to administer lipid-containing analog instead of molnupiravir in COVID-19 therapeutic protocols. In this respect the authors reported the successful synthesis catalyzed by bacterial phospholipase D of 5′-dimyristoyl derivative of N4-hydroxycytidine – the compound allegedly more efficient than molnupiravir in inhibiting SARS-CoV-2 replication.

Keywords: COVID-19 pandemic, chemotherapy, modified nucleoside, N4-hydroxycytidine, chemical and enzymatic synthesis of molnupiravir, phospholipase D, transphosphatidylation

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МОЛНУПИРАВИР КАК ПЕРСПЕКТИВНОЕ ПРОЛЕКАРСТВЕННОЕ СРЕДСТВО ДЛЯ ТЕРАПИИ COVID-19

Аннотация. Непрекращающаяся пандемия COVID-19, сопровождающаяся появлением все новых антигенных вариантов возбудителя, делает проблематичным подход, основанный на применении для борьбы с SARS-CoV-2 только профилактических вакцин. Это обстоятельство обусловливает настоятельную необходимость скорейшего поиска и создания химиопрепаратов прямого противовирусного действия.

В настоящей мини-обзорной статье приводится информация о химических и ферментативных методах получения молнупиравира, считающегося на сегодняшний день одним из самых перспективных лекарственных средств для терапии COVID-19. В заключении литературного обзора предлагается использовать для терапии COVID-19 не сам молнупиравир, а его липидсодержащий аналог. В связи с этим авторы сообщают о синтезе ими с помощью бактериальной фосфолипазы D 5′-димиристоильного производного N4-гидроксицитидина – соединения, которое, как предполагается, более эффективно, чем молнупиравир, в отношении ингибирования репликации SARS-CoV-2.

Ключевые слова: пандемия COVID-19, химиотерапия, модифицированный нуклеозид, N4-гидроксицитидин, химический и ферментативный синтез молнупиравира, фосфолипаза D, трансфосфатидилирование

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Introduction. COVID-19 pandemic caused by novel coronavirus of morbid acute respiratory syndrome (SARS-CoV-2) turned into the grave global challenge for the health of human population. The estimates in early May 2022 recorded over 516 mln people around the world infected with the virus, with overall death toll taking 6.25 mln lives [1].

Specific vaccination is known to be the most effective means to control viral diseases. Numerous pharmaceutical companies joined into the race to clinch the championship in design of vaccine against SARS-CoV-2. However, the probability cannot be ruled out that vaccines opposing rapidly mutating

coronavirus will tend to lose their efficiency. This argument substantiates the efforts in favor of formulating medicines displaying direct antiviral activity [2, 3].

Since the development of specific preparations for treatment of every newly appearing infection is a long-term process taking several years even in the most favorable circumstances, in the meantime the medical community is focused on the existing registered broad-spectrum antiviral trademarks. The previous experience in curing infections of viral origin is conveyed to the foreground for public debates.

The chemical core of the most renowned antiviral preparations is made up by modified nucleosides, yet, they do not possess therapeutic activity per se. To transform into the active form, they undergo multistage intracellular metabolic activation consisting in step-by-step conversion of nucleoside into nucleoside-5′-triphosphate. The initial stage (5′-monophosphorylation of nucleoside) is often the step limiting activation rate. Moreover, the total lack of enzyme nucleoside kinase responsible for this stage, completely blocks the conversion of nucleoside into the active substance.

To by-pass this stage the so-called pro-Tide strategy is applied, envisaging in one of the variants substitution of 5′-phosphatidyl derivatives for the respective nucleosides [4]. Penetrating into the cells, the above-mentioned compounds are hydrolyzed under the impact of cellular esterases, turning immediately into nucleoside-5′-monophosphates (without mediation of nucleoside kinases).

Thus, generation of active substance (with concomitant therapeutic effect) may happen even in cells deficient in nucleoside kinases and, as a consequence, resistant to action of the parent nucleoside.

Of special interest in this respect are reports circulating in periodicals of therapeutic properties of molnupiravir-antiflu preparation with commercial codes MK-4482 and EIDD-2801. This prodrug is composed of nucleoside carcass based on N4-hydroxycytidine [5, 6] – a modified nucleoside originally synthesized in Emory University, private research institution located in Atlanta, Georgia, USA.

Now molnupiravir is promoted by Merck pharma company as a new peroral antiviral remedy for COVID-19 [7]. Animal trials demonstrated beneficial effect of molnupiravir in preventing virus transmission and inhibiting SARS-CoV-2 activity [8].

Structurally molnupiravir is 5′-isobutyl derivative of antiviral ribonucleoside of direct action – EIDD-1931 (or β-D-N4-hydroxycytidine).

In blood plasma molnupiravir is split to release EIDD-1931. Subsequently it is phosphorylated by kinases in host cells to the respective 5′-triphosphate as the active antiviral agent [9, 10] (Fig. 1).

The animal test models of infections caused by various coronaviruses, flu virus, Ebola virus have shown that EIDD-1931 successfully inhibited replication of many RNA-containing viruses [10, 12, 13]. This peroral preparation proved high efficiency against SARS-CoV-2 infection and adequate safety level [14].

Clinical trials at phase 1 revealed safety of molnupiravir as a novel peroral antiviral drug and good response (no side effects) in healthy volunteers [15]. During phase 2 of clinical trials (multi-centered, randomized, placebo-controlled, double blind control study) the patients with light and medium gravity of the disease were administered molnupiravir twice per diem in the course of 5 days.

The results evidenced that the tested drug reduced the level of SARS-CoV-2 transcription, accelerated the rate of virus elimination, prevented COVID-19 progression and replication of SARS-CoV-2 [14, 16].

The findings collected from synchronized phases 2 and 3 indicated attractive prospects of molnupiravir to become the recognized therapeutic agent for non-hospitalized patients suffering from COVID-19 [11].

Metabolism of molnupiravir. Molnupiravir is peroral 5′-modified analog of N4-hydroxycytydine originally conceived (in 2019) to cure influenza. Surprisingly, the drug displayed elevated activity to counter SARS-CoV-2 under laboratory conditions and in model experiments with test animals [8].

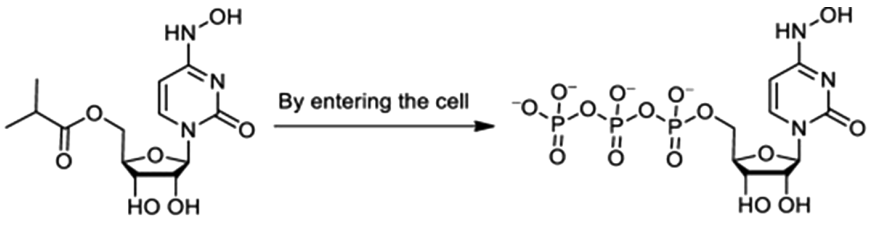

Fig. 1. Metabolic activation of molnupiravir [11]

It should be noted that compounds with a broad spectrum of antiviral activity, including inhibition of SARS-CoV-2 replication, were pointed out among numerous modified analogs of natural nucleosides and nucleotides. Some of them are passing clinical trials to check COVID-19 treatment efficiency [11]. However, in contrast to similar preparations authorized for urgent control of COVID-19, large-scale production of molnupiravir may be arranged. The product requires no special terms for storage, and peroral administration mode enables to apply it on off-ward basis. Molnupiravir successfully passed all 3 phases of clinical trials revealing no significant side effects [17]. The drug dose sufficient and safe for the patients infected with coronavirus was established experimentally by S. H. Khoo et al. [18]. The patients divided in groups 6 persons each were fed perorally 300, 600 and 800 mg of molnupiravir twice per diem during 5-day course. Following examination of 103 participants the authors stated no drug rejection in the tested dosages, hence it was considered as safe. Judging from these results, it was recommended to prescribe 800 mg dose twice a day to achieve the maximum therapeutic effect.

Mechanism of molnupiravir action. Apprehension of the mechanism of action of any medicine on the molecular level is known to play a crucial role in direction (concentration) of research efforts for further elaboration of antiviral compounds. It was found that molnupiravir molecule getting inside the cell undergoes metabolic activation resulting in synthesis of 5′-triphosphate derivative of N4-hydroxycytidine. Later RNA polymerase (the enzyme catalyzing replication of RNA matrix) incorporates the corresponding modified mononucleotide into viral RNA genome. Any molecular event of this kind may induce mutation and accumulation of such mutations blocks propagation of the virus [19] (Fig. 2).

In line with the above-illustrated mechanism of action molnupiravir likewise triggers generation of "life-incompatible" mutations in other RNA-containing viruses interfering with their reproduction [6, 8, 20, 21]. Biochemical and structural investigations have elucidated that viral RNA-dependent RNA polymerase recognizes metabolically activated molnupiravir as the analog of natural cytidine-5′-triphosphate. The enzyme then engages the resulting RNA chain fragment as the matrix for copying, and modified cytosine is linked either with guanine or adenine resulting in emergence of mutant daughter RNA. Despite probable adverse mutagenic consequences, molnupiravir is distinguished by superb pharmaco-kinetic characteristics evident in the course of peroral administration.

Methods of molnupiravir synthesis. The pioneering approach to production of molnupiravir was proposed by researches of Emory University [23]. In conformity with the devised scheme (Fig. 3) molnupiravir was synthesized in 5 stages, starting from uridine (4). The product yields from the final 2 stages were not specified but in general the procedure attained the top yield of the end product 17 %.

Initially hydroxyl groups adjoining the second and third carbon atoms of ribose molecule were protected with acetone at room temperature. Afterwards the compound (5) was supplemented with 4-(N,N-dimethylamino)-pyridine, triethylamine and isobutyric anhydride (6). The resulting compound (7) was dissolved in acetonitrile, then triethylamine and 1,2,4-triazole were added. The obtained solution was treated with phosphoryl chloride and cytidine derivative (8) modified with triazole was recovered with the yield 29 %. The compound (8) was solved in 2-propanol and treated with hydroxylamine to produce compound (9) (yield 60 %). Using formic acid the authors removed the protective shield from hydroxyl groups at 2′- and 3′-C positions at ambient temperature and synthesized molnupiravir (10).

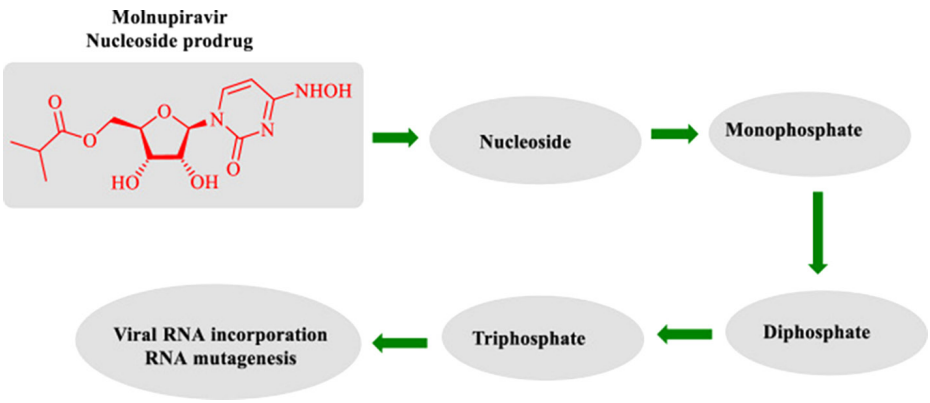

Fig. 2. Mechanism of antiviral action of molnupiravir [11]

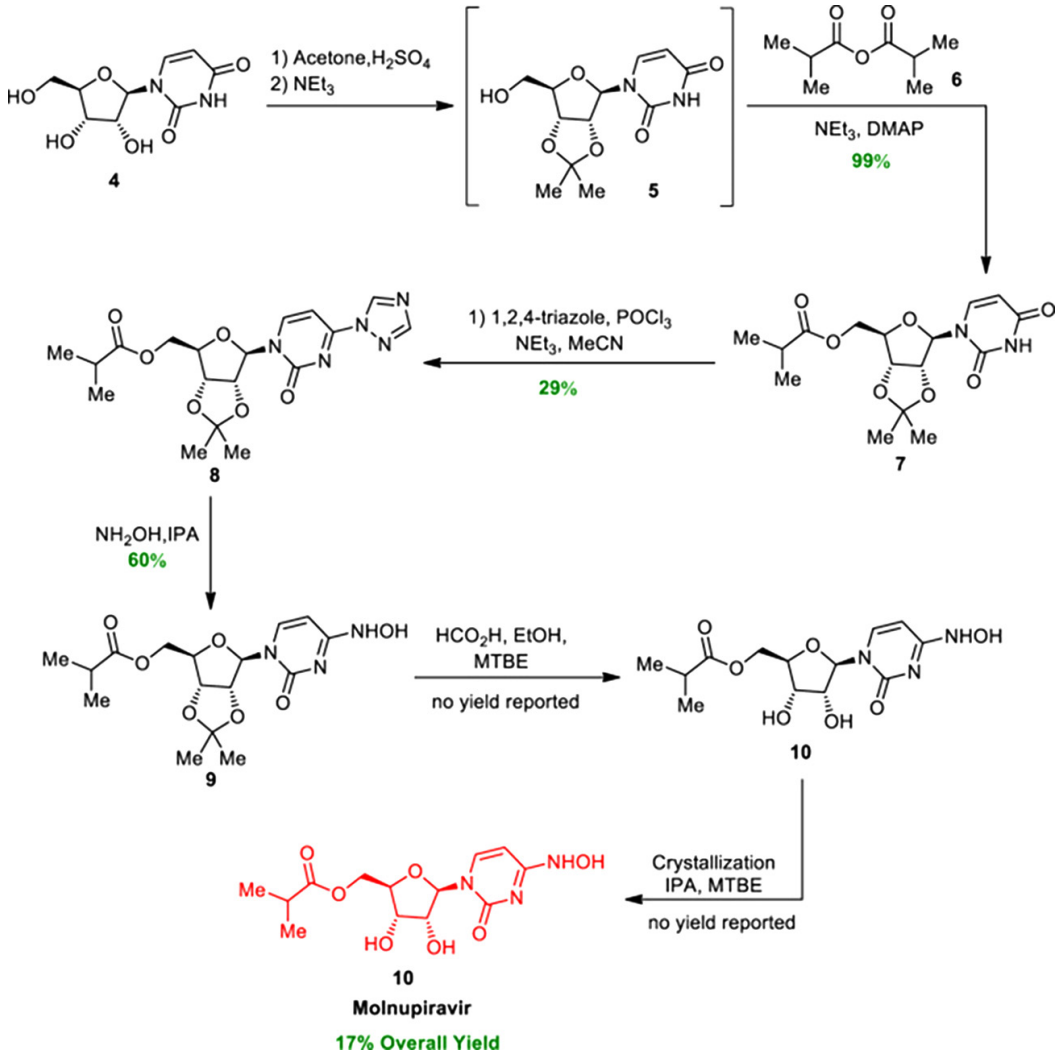

Fig. 3. Scheme of molnupiravir synthesis originating from Emory University [23]

A. Steiner et al. [24] considerably upgraded the chemical process of manufacturing molnupiravir from uridine by simplifying isolation of the end product and its purification. They succeeded in lifting the final yield from 17 to 61 % as compared with the prototype method [23].

Four-stage molnupiravir synthesis from cytidine was conducted in one reaction vessel by T. Hu et al. [25]. This method utilized dimethylacetal N,N-dimethylformamide as the selective protective agent to ensure ready site-specific esterification of 5′-hydroxyl group of nucleoside. Noteworthy that the authors of the cited research replaced more expensive uridine with less costly cytidine. The ultimate yield of the target product equaled 70 %.

V. Gopalsamuthiram et al. [26] also produced molnupiravir from cytidine in 4 stage process on a preparative scale (in gram amounts). The distinction of the proposed chemical technology is that it envisages application of cheap reagents and relatively eco-friendly solvents, like isopropanol, acetone, acetonitrile and water. The authors reached molnupiravir yield 36–41 % and purity grade around 98 %.

The latest decades are known to witness the clear-cut trend of transition from the conventional chemical processes to the so-called green chemistry [27] principles opting for ecologically safe enzymatic reactions in preference over more hazardous chemical technologies.

In this regard a series of papers describing chemical-enzymatic methods of molnupiravir production deserves special attention. For instance, T. Benkovics et al. [28] outlined the scheme of molnupiravir synthesis from ribose and uracil engaging three biocatalysts and available reagents. This biosynthetic procedure is triggered by selective enzymatic (*Candida antarctica* lipase Novozym 435) esterification of primary alcohol group in ribose molecule using isobutyric anhydride as the donor of isobutyl residue. It is followed

by adjoining phosphate to ribose 1-OH group mediated by kinase. Further on uridine phosphorylase catalyzes nearly ideal conversion of intermediate into molnupiravir not aggravated by significant release of by-metabolites. The overall yield of the end product upon recovery from the reaction mixture was 69 %.

N. Vasudevan et al. [29] developed two-stage chemical-enzymatic method of molnupiravir synthesis comprising esterification by lipase from *Candida antarctica* (Novozym 435, Denmark) and hydroxamination of cytidine. The authors used relatively accessible nucleoside cytidine as the initial substrate. As a result, 75 % yield of the target compound was attained.

G. P. Ahlqvist et al. [30] rationalized and scaled up the process described in the previous study [29]. They carried out preparative synthesis of molnupiravir (dozens of grams) from cytidine and its purification, not resorting to laborious chromatography. Transamination was applied at the first stage, while the required drug was derived by selective enzymatic acylation. In general, molnupiravir of pharmaceutical purity grade (97–99 %) was produced with the final yield 41 %. This approach proved more labor-saving and less costly then the previous analytical variant [29].

D. J. Paymode et al. [31] reported 2-stage procedure of molnupiravir synthesis from cytidine by direct hydroxamination of cytosine ring and esterification of 5′-primary hydroxyl group in carbohydrate moiety of nucleoside. Both reactions were terminated with 90 % yield. The total yield of isolated compound rose by 23 % (from 37 to 60 %) in case of product purification by crystallization rather than chromatography. Eco-safe solvents, e.g. water and 2-methyltetrahydrofuran were used in the process. In accordance with methodology described in the earlier investigations [29] and [30] acylation was performed with the aid of commercial lipase preparation.

Several literature sources deal with production of N4-hydroxycytidine as an essential intermediate of molnupiravir synthesis. The most impressive technique was proposed by A. Burke et al. [32]. N4-hydroxycytidine was produced by one-stage biocatalytic transformation of cytidine using recombinant cytidine deaminase (E.C. 3.5.4.5) with activity modified by insertion of mutation T123G near the active site of the enzyme. As a consequence, selectivity of the enzyme was raised resulting in reduced amount of by-metabolite (uridine) and 4.9 g output of the end product from 5 g of cytidine (the total yield 85 % with the purity grade over 98 %). The researchers realized the conceived approach in the process manufacturing N4-hydroxycytidine on the scale of hundreds of grams of the target product.

Synthesis of 5′-phosphatidyl derivative of N4-hydroxycytidine. Metabolic activation of molnupiravir (5′-isopropyl ester of N4-hydroxycytidine – EIDD-2801) upon introduction into the human body includes 4 enzymatic stages: a) removal of isopropyl residue from the molecule; b) transformation of the resulting nucleoside into nucleoside-5′-monophosphate; c) phosphorylation of nucleoside-5′-monophosphate to nucleoside-5′-diphosphate; d) conversion of nucleoside-5’-diphosphate into the corresponding triphosphate.

In our opinion, it seems expedient to engage as the drug to control SARS-CoV-2 coronavirus infection instead of molnupiravir 5′-phosphatidyl derivative of its nucleoside moiety – N4-hydroxycytidine, i.e. EIDD-1931. We presume that in this case the initial stage of activation (under the impact of esterase) should lead directly to generation of nucleoside-5′-monophosphate, passing by the most critical stage of nucleoside monophosphorylation catalyzed by nucleoside kinase.

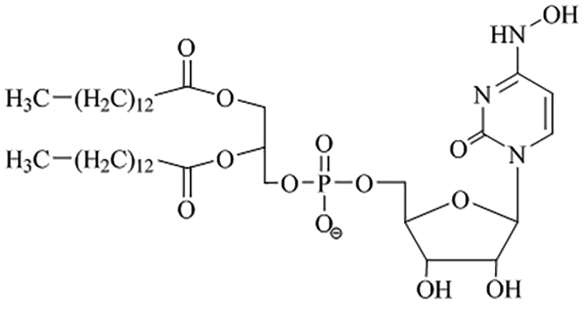

Fig. 4. Molecular structure of 5′-dimyristoylphosphatidyl analog of N4-hydroxycytidine

We tested the feasibility of using phospholipase D from strain *Streptomyces netropsis* BIM B-428D deposited in Belarussian collection of nonpathogenic microorganisms, Institute of Microbiology, National Academy of Sciences of Belarus and capable to transfer phospholipid residue from lecithin to primary alcohols in the process of producing 5′-phosphatidyl derivative of N4-hydroxycytidine.

N4-hydroxycytidine was synthesized in compliance with the methodology developed by N. Vasudevan et al. [29]. Synthesis of 5′-phosphatidyl-N4-hydroxycytidine (Fig. 4) was accomplished in biphasic reaction mixture of 1 ml volume composed

of chloroform and aqueous buffer phases in 2:1 ratio. The mix contained nucleoside, 1,2-dimyristoylphosphatidylcholine and dry preparation of phospholipase D. The reaction proceeded at 37 °C during 6 h. Under these conditions the conversion of nucleoside into the end product exceeded 70 % [33].

The target compound gives a positive color reaction (is stained blue) upon interaction with specific reagents to phospholipids and amino groups. Its spectrum in UV-range matches the spectrum of initial nucleoside (λ max = 238 nm). The chemical structure of the compound is confirmed by the method of ^{1}H-NMR-spectroscopy.

Conclusion. Nowadays COVID-19 pandemic turned into the global scourge taking annually a plenty of human lives. Many experts representing diverse branches of science, like organic and biological chemistry, pharmacology, molecular biotechnology, etc. have focused their efforts on research and development of medicinal agents effective against emerging threat of coronavirus SARS-CoV-2.

Molnupiravir as a peroral antiviral drug demonstrated attractive prospects in treatment of out-patients suffering from COVID-19. Clinical trials at phases 1, 2 and 3 showed that molnupiravir considerably reduced the risk of hospitalization and lethality in adults with light and moderate progression of COVID-19 pathology. Thus, the tested drug may play a vital role in control of SARS-CoV-2 infection.

The authors of the presented review succeeded in the first synthesis of 5′-phosphatidyl derivative of N4-hydroxycytidine. Such modification of molnupiravir is assumed to shorten the pathway of metabolic nucleoside transformation into the corresponding nucleoside-5′-triphosphate and hence is likely to increase activity to withstand pandemic coronavirus.

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