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L-ASPARAGINASES AND THEIR POTENTIAL IN BIOTECHNOLOGY AND MEDICINE

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L-Asparaginase (EC 3.5.1.1) is a key enzyme that hydrolyzes L-asparagine to L-aspartic acid and ammonia. This feature of L-asparaginase is used in anti-cancer therapy to inhibit protein synthesis in cancer cells. Therefore, L-asparaginase is used as a basis for chemotherapy to treat patients with acute lymphoblastic leukemia in pediatrics. Commercial L-asparaginases for healthcare applications are mainly obtained from *Escherichia coli* and *Erwinia chrysanthemi* (renamed to *Dickeya dadantii*). However, the high prevalence of adverse effects complicates the long-term clinical use of L-asparaginase, and therefore current research focuses on the search for new enzymes or on modifying the properties of enzymes already known. At the same time, L-asparaginase has become indispensable for the food industry in the recent years, when it had been recognized as one of the possible tools for removing L-asparagine from foods that are at risk of acrylamide formation during thermal processing. This review provides an overview of the current use of L-asparaginase and its pitfalls.

Keywords: L-asparaginase, acute lymphoblastic leukemia, acrylamide, biosensor, biological anti-cancer treatment

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1. Introduction

L-Asparaginases (EC 3.5.1.1) (L-ASNases) are enzymes classified as amidohydrolases. Their main function is the hydrolysis of L-asparagine to L-aspartic acid and ammonia (Scheme 1). The presence of L-ASNases has been confirmed in many microorganisms, plants, and animals. On the basis of amino acid sequence, biochemical properties, and structure, L-ASNases are divided into three families: bacterial (type I and type II), plant (type III), and rhizobial[1], however, this classification no longer corresponds to all current knowledge and it can be assumed that a new nomenclature will be established in the future[2].

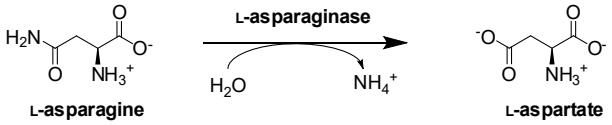
Thanks to their ability to cleave L-asparagine, L-ASNases are widely used in the treatment of cancer as well as in the food industry to decrease the amount of acrylamide. Recently, their use in biosensors[3] has received increasing attention and their potential application in the treatment of infectious diseases[4] has also been tested.

2. Use of L-asparaginases in medicine

So far, L-ASNase has found its greatest application in medicine, in the treatment of childhood acute lymphoblastic leukemia (ALL) and other hematological malignancies[5]. In recent years, however, information has emerged suggesting the potential of these enzymes in other areas of medicine, specifically in the treatment of certain autoimmune[6] and infectious diseases[7,8].

2.1. Cancer treatment

L-ASNase is used in combination with other drugs primarily to treat various lymphoproliferative disorders,



Scheme 1. **L-Asparaginase catalyses the chemical conversion of L-asparagine to L-aspartic acid and ammonia**

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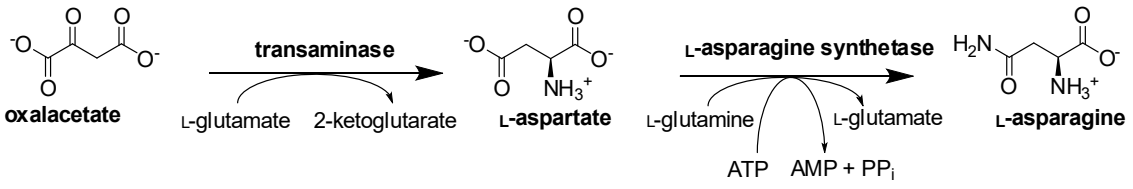
especially ALL. ALL is one of the malignancies during which there is excessive production of immature leukocytes, resulting in suppression of the development of other blood cells as well as the spread and deposition of these tumor cells to other organs such as the liver, spleen, and lymph nodes[5]. In addition to ALL therapy, L-ASNase is used to treat Non-Hodgkin's lymphoma[9], Hodgkin's disease[10], chronic lymphocytic leukemia[9], lymphosarcomas[10], acute myeloid leukemia[10], and acute myelomonocytic leukemia[9]. Its efficacy has also been reported in the treatment of solid tumors such as pancreatic and prostate carcinoma[11], reticulum sarcoma[10], lung adenocarcinoma[9], melanosarcoma[9], ovarian cancer[11], and some brain tumors[9,11]. For completeness, it may be added that this enzyme has also found application in veterinary practice, for example in the treatment of cattle with bovine viral leukosis[12]. These L-ASNase therapies take advantage of the fact that L-asparagine is one of the proteinogenic amino acids essen tial for protein biosynthesis and other cellular processes. Although it is a non-essential amino acid and is directly synthesized in the human body, many types of leukemic immature cells are sensitive to low levels of L-asparagine in the external environment[13,14]. This is because neoplastic cells, in contrast to healthy cells, have low production of the enzyme L-asparagine synthetase, and thus lack the ability to synthesize L-asparagine in sufficient quantities to meet the needs of rapid growth and uncontrolled proliferation and fail to respond quickly enough to L-asparagine deprivation[15,16]. It is reported that blood levels of L-asparagine are most commonly maintained in the range of 40–80 μmol L^{-1} (ref.[17]), but may range from units to hundreds of μmol L^{-1} in healthy individuals. In contrast, in patients with ALL not treated with L-ASNase, L-asparagine level rises from units to hundreds of mmol L^{-1} (ref.[18,19]). The effect of appropriate L-ASNases can reduce blood L-asparagine concentrations to less than 0.1–3 μmol L^{-1} (ref.[17,20]), leading to nutritional deprivation, cessation of proteosynthesis and proliferation, and subsequently apoptosis of leukemia cells[21].

The *de novo* synthesis of L-asparagine in healthy cells begins via enzymatic transamination (Scheme 2). The precursor of L-asparagine biosynthesis is oxalacetate, which reacts with L-glutamine in the presence of transaminase to form L-aspartate and 2-oxoglutarate. L-Aspartate then reacts with L-glutamine to form L-asparagine in an ATP-dependent reaction that is catalyzed by L-asparagine synthetase[22]. The above information suggests that sufficient activity of L-asparagine synthetase is also related to a sufficient source of ATP (ref.[14,23]). In this context, it is necessary to highlight the selective action of L-ASNase on cancer cells compared to the non-selective action of other therapeutics[20].

In terms of medical applications, the most important bacterial L-ASNases are currently type II ASNases, which are typically characterized by high affinity for L-asparagine and relatively low affinity for L-glutamine[2]. Although these enzymes have been used in cancer treatment for over 40 years, preparations containing L-ASNases from only two bacterial sources are available on the pharmaceutical market, namely, preparations containing native L-ASNase from *Escherichia coli* (native and pegylated forms) and from *Erwinia chrysanthemi* (hereafter referred to as *Dickeya dadantii*, renamed in 2005 by the Laboratory of Genomic Evolution at the University of Wisconsin[24]). Due to the bacterial origin of these enzymes, their use is associated with several adverse effects, as will be described in the following section.

One of the critical characteristics for the possibility of effective use of L-ASNase in the treatment of ALL is substrate affinity. Thus, in simplified terms, it is necessary that the value of their Michaelis constant (K_M) of a reaction using L-asparagine as a substrate is in the order of μmol L^{-1} (see L-asparagine blood concentration). This is fulfilled by the two enzymes mentioned above, namely the K_M value for L-ASNase from *E. coli* was determined to be 15 μmol L^{-1} (ref.[25]), and for the enzyme from *D. dadantii,* it was determined to be 58 μmol L^{-1} (ref.[26]).

Important for the discovery of the antineoplastic effects of L-ASNase was the year 1922 (ref.[27]), in which the presence of L-ASNase in a guinea pig serum was detected. This L-ASNase was the first successfully tested in children suffering from ALL in 1966 (ref.[28]). The first drug approved by the US Food and Drug Administration (FDA) for the treatment of ALL was Elspar® in 1978, which contained native L-ASNase isolated from *E. coli*[29,30]. However, it is now unavailable as it has been withdrawn from the market due to manufacturing problems and new stricter drug approval conditions[31]. In 1985, a drug with native L-ASNase from *D. dadantii* (Erwinase)®[32] was approved, and in 1994, Oncaspar® was approved containing L-ASNase conjugated to polyethylene glycol (PEG) (so-called pegylated L-ASNase). This resulted in a lower immunogenicity of this enzyme and a longer biological half-life (ref.[33–36]). An overview of other preparations that can be encountered on the world market today is given in Table I.



Scheme 2. **Mechanism of *de novo* synthesis of L-asparagine**

Table I
Overview of commercial drugs containing L-asparaginase

Origin of the enzyme	Trade name of the preparation (form)	Approval year, institution, region	Note
Escherichia coli	Elspar® (ref.[30]) (native)	1978, FDA, USA	Production ended in 2012
	Kidrolase® (ref.[77]) (native)	1974, France	Biosimilar form of Elspar®
	Oncaspar® (ref.[35]) (pegylated)	1994, FDA, USA 2016, EMA, Europe[a]	FDA recommended for first-line use since 2006
	Leunase® (ref.[78]) (native)	1971, PMDA (since 2004), Japan	Biosimilar form of Elspar®, produced in a modified strain of *E. coli*[79] since 2013
	Spectrila® (ref.[80]) (native, recombinant)	2015, EMA, Europe[a]	
	Asparlas® (ref.[81]) (pegylated)	2018, FDA, USA	
Dickeya dadantii	Erwinase® (ref.[82,83]) (native)	1985, MHRA, UK 2011, FDA, USA	For patients exhibiting hypersensitivity to L-ASNase from *E. coli*
	Rylaze® (ref.[84]) (native, recombinant)	2021, FDA, USA	Produced in *Pseudomonas fluorescens*

[a] For simplicity, the table uses Europe as the region for the European Medicines Agency (EMA), but the EMA is actually valid for the countries of the European Union and namely Iceland, Liechtenstein and Norway

Currently, the treatment of ALL is a very complex process that involves, within chemotherapy, 6 or more drugs[37]. Therapy involves the administration of L-ASNase from *E. coli* or *D. dadantii*[38] in combination with other drugs such as vincristine or dexamethasone (depending on the stage of treatment)[39] and in combination with radiotherapy[40].

2.2. Side effects

Treatment with L-ASNase can lead to many serious side effects. The severity of the side effects can vary considerably from patient to patient and depends, among other things, on whether treatment with other drugs or radiotherapy is being given at the same time. The most common side effects include allergic reactions, nausea, fever, and central nervous system toxicity[38,41].

Many of these adverse effects have been attributed to the fact that these enzymes can use also L-glutamine as a substrate in addition to L-asparagine[1,42]. L-Glutamine is the major transport source of ammonia for many biosynthetic reactions, and so a long-term decline of L-glutamine level in plasma disrupts biochemical function, particularly in the liver[25]. These side effects often make it impossible to complete the entire treatment process leading to remission[43]. However, the question arises whether the therapeutic index (the ratio of the dose of the drug causing toxicity to the dose of the drug causing the therapeutic effect) of L-ASNase could be increased by reducing or completely eliminating the L-glutaminase activity of the enzyme used, or whether this would also proportionally reduce the anticancer effect. In fact, it is possible to found theories in the literature that, on the contrary, speak of a supportive therapeutic effect of L-glutaminase activity, since, under the action of L-asparagine synthetases, L-glutamine is an essential amino group donor for the synthesis of L-asparagine[22]. Chan W. K. et al.[43] formulated a theory that some cancer cells are sensitive to the absence of L-asparagine due to the absence of L-asparagine synthetases, thus L-glutaminase activity does not support the antineoplastic effect and it is advisable to use L-ASNase with the lowest L-glutaminase activity. However, if it is a type of tumor in which the cells have active L-asparagine synthetase but is sensitive to the absence of L-asparagine, L-glutaminase activity will support the antineoplastic effect of L-ASNase[43].

For completeness, it should be mentioned that the enzymes used in current practice exhibit L-glutaminase activity in addition to L-asparaginase activity (the K_M values of the reaction using L-glutamine as a substrate are for *E. coli* and *D. dadantii* L-ASNases 3.7 mmol L^{-1} and 10.3 mmol L^{-1}, respectively (ref.[44])). The concentration of L-glutamine in the blood is approximately 0.5–0.8 mmol L^{-1} (ref.[45]). However, further research is

undoubtedly necessary to draw a clear conclusion about the presence of L-glutamine activity in drugs.

Another widely discussed problem with the use of the aforementioned L-ASNases in medicine is their foreign origin. All drugs on the market contain L-ASNase originating from bacterial sources and can therefore cause an adverse immune system reaction in the human body, leading to the inability of L-ASNase to be used for a long time or, in some cases, even to complete the treatment already in progress. One previously mentioned possibility of reducing the immunogenicity of native L-ASNase is to mask its structure for the immune system by attaching PEG molecules that reduce the ability of macrophages to recognize the enzyme, thus leading to a slower degradation of the administered enzyme[44,46]. The reaction of antibodies with the antigenic structures of the protein may not only trigger an exaggerated reaction of the immune system, but only silently deactivate the protein, rendering treatment with L-ASNase ineffective and resulting in resistance to the given L- ASNase (ref.[47]). In addition, only two sources of L-ASNases are commercially approved (see above), which represents a very limited selection that should be expanded in the future to include new L-ASNases from other sources or their designed mutant forms with sutaible properties.

2.3. Other medical applications

In addition to its long-standing anticancer activity, the potential use in other medical applications has recently been described for L-ASNases[10]. For example, published studies are highlighting the potential of L-ASNases in the treatment of infectious and autoimmune diseases[48].

Streptococcus pyogenes, a bacterium belonging to a group referred to as "Group A Streptococcus" (GAS), has been studied in relation to the potential use of L-ASNases in the treatment of infectious diseases. This bacterium causes diseases of varying severity[7]. Upon adherence to the host cell, GAS produces toxins (streptolysins) that cause endoplasmic reticulum stress, which is manifested by, among other things, increased expression of the gene for L-asparagine synthetase, resulting in increased production of the amino acid L-asparagine, which induces GAS-proliferation and growth. Without this amino acid, approximately 17 % of GAS genes are not expressed (ref.[49]). Here the use of a drug containing L-ASNase was proposed, which has been shown to suppress the growth of the GAS pathogen in a mouse model of human bacteremia (bacteria present in the bloodstream) and also in human blood[49]. Other pathogenic bacteria, such as *Clostridium botulinum*, *Listeria monocytogenes,* or *Staphylococcus aureus,* also contain streptolysin-like toxins. Thus, it is possible that preparations with L-ASNase could also be effective in the treatment of other infectious diseases[4].

Several studies have already confirmed the ability of bacterial L-ASNases to influence the human immune response, specifically their immunosuppressive and anti-inflammatory effects[50]. Initial trials have also been conducted suggesting the possibility of using pegylated L-ASNase from *E. coli* in the treatment of autoimmune diseases. In this case the studied disease was rheumatoid arthritis, the treatment of which was tested in a mouse model of collagen-induced arthritis[6]. It was shown that administration of this L-ASNase suppressed the B-lymphocyte immune response mediated by helper T-lymphocytes. In the humoral immune response, a lower amount of antibodies formed by B-lymphocytes was observed[10], which is consistent with the finding that *Salmonella typhimurium* virulence is mediated by its L-ASNase. However, the mechanism of this action has not yet been fully elucidated. The extracellular domain of the T-lymphocyte receptor contains a variable portion corresponding to the antigenic structure, which is most often composed of a heterodimer of alpha and beta chains. It has been found that L-ASNase from *S. typhirum* prevents modulation of the beta chain, thereby reducing the T-lymphocyte response[51].

In one study, even antiviral effects of L-ASNase were described, specifically in Coxsackie B3 virus, where L-ASNase isolated from *Spirulina maxima* algae prevented the adsorption of the virus to the cell surface and its penetration inside by a yet unexplained mechanism[8].

3. Use of L-asparaginases in the food industry

The L-ASNases have also found their application in the food industry, specifically in reducing the amount of acrylamide in heat-processed foods. The neurotoxic effects of acrylamide have been known for more than half a century[52] and it has also been identified as a potential carcinogen for decades[53,54]. However, until the accident in Sweden (1997), in which large amounts of acrylamide were accidentally released into the environment during the construction of a railway tunnel, acrylamide was not associated with food. Water contamination, the death of a large number of animals, media interest, and public panic led first to a witdrawn of the food from that area and subsequently to an interest of monitoring acrylamide in food. However, this has led to the discovery that some foods outside the area are containing higher levels of acrylamide too. Scientific research has gradually shed light on the mechanism by which acrylamide is formed in food and since 2002 efforts have been made, in cooperation with organizations such as the World Health Organisation (WHO), the European Food Safety Authority (EFSA), and the Food and Agriculture Organisation (FAO), to limit the amount of acrylamide in food through legislation in which different recommendations on the processing of raw materials and specific limits on acrylamide content apply to each commodity[55].

The essence of the use of L-ASNase in the food industry is therefore to reduce the concentration of L-asparagine, which occurs naturally in food and can (under certain conditions) serve as a precursor for the for-

mation of acrylamide – a neurotoxin and potential carcinogen[21,56,57]. The risk of acrylamide formation exists in carbohydrate-rich foods processed at high temperatures (usually above 120 °C) and low humidity, such as French fries, potato chips, bakery products, breakfast cereals, and roasted coffee[5,58]. In a process called the Maillard reaction, L-asparagine reacts with reducing carbohydrates to form acrylamide in a sequence of reactions. At the same time, however, the Maillard reaction is used to produce a number of other compounds which are in turn desirable because of their sensory properties[58].

Currently, the food business operators in Europe are required to comply with Commission Regulation 2017/2158, which sets out measures to reduce the presence of acrylamide in food[58]. There is a number of approaches that can reduce the amount of acrylamide in final products. These include selecting appropriate raw materials, adding a range of different additives, or changing production conditions such as temperature or pH. However, the use of these methods often results in a change in the quality of the final product, which is apparent in its taste or appearance. The advantage of using L-ASNases is seen by many experts as the fact that the desired product characteristics are not adversely affected by the use of this enzymatic method. At the same time, the application of L-ASNases have demonstrated that the amount of acrylamide can be, in some cases, reduced by up to 90 % (ref.[59]).

As examples of the practical use of L-ASNases in the food industry, we would like to mention the commercially available products of both the PreventASe® and Acrylaway® series, which contain L-ASNases originating from *Aspergillus niger* and *Aspergillus* oryzae, respectively[21].

The products under the PreventAse® label are manufactured by DSM from the Netherlands. The optimum pH for the activity of these products is in the more acidic range (pH 4–5), and the temperature optimum for the preparation is approximately 50 °C. The ability of the product to cleave L-asparagine and thereby reduce the amount of acrylamide formed has been described in several studies with different food products[60–62]. Treatment of a wheat-oat loaf of bread with this product for 15 min at 32 °C before baking resulted in a 46% reduction in the amount of acrylamide formed (ref.[60]). Rotmann et al.[61] decided to study the use of L-ASNase to reduce the amount of L-asparagine in the preparation of French fries according to industry standards. PreventAse L® showed the best properties for the procedure. The product was applied after the blanching step at different concentrations and left to act for 1 min at 60 °C. As a result, acrylamide was reduced by up to 59 % (ref.[61]). The potential of the product from the Netherlands has also been recently studied in the preparation of pizza dough[62]. In this work, different formulations of PreventAse® under the labels M, W, and XR-BG were used and added to the dough. In this case, L-ASNase was active throughout the kneading of the dough (for hours). When applying the XR-BG formulation (developed for use in environments with higher pH values), the formation of acrylamide was reduced by about 89 % due to the cleavage of L-asparagine, and after the addition of preparations M and W, the acrylamide content was reduced even below the detectable limit[62].

Acrylaway® is manufactured by Novozymes A/S from Denmark. The enzymes contained in these products show the highest activity at pH 6-7 and at a temperature of around 60 °C. The effects of these products have also been described in several studies[63–65]. Similar to PreventAse®, the product was tested in the preparation of French fries after the blanching step[63]. In this study, a 60% reduction in the amount of acrylamide formed was achieved after exposure to the enzyme preparation at 40 °C for 20 min. The work of Porto et al. is an example of a study dealing with the effect of Acrylaway® on the amount of L-asparagine in coffee beans[64]. The treatment of coffee beans resulted in about 30% reduction of L-asparagine content. Due to experiments in another study also aimed at reducing the L-asparagine content of coffee beans, a decrease in acrylamide content of up to 77 % was achieved after coffee roasting (ref.[65]). Both of these studies show that it is possible to reduce the L-asparagine content even in such solid matrices as coffee beans, but a pre-treatment step is necessary to increase the availability of L-asparagine for L-ASNase (hot steam pre-treatment of the beans was chosen in both works).

In addition to the application of purified enzyme alone, a whole cell approach has been proposed. Kerry company, in collaboration with Rennaissance BioScience Corp. came to market in 2019 with Acryleast®, a product containing baker's yeast[66]. In 2022, the company launched a product called Acryleast Pro® showing more than twice the L-asparaginase activity of the original product[67]. This yeast was bred through adaptive evolution to produce a variant with the L-asparagine activity that would work under food preparation conditions (e.g., dough proofing). Normally, yeasts do not cleave the L-asparagine present in raw materials as they use more available nitrogen sources[68]. Of course, the use of the activity of this whole cell-containing preparation is only possible for the preparation of food products that use the activity of baker's yeast. At the same time, it is a very elegant method that does not require changes in the production process and does not affect the taste or other properties of the final product.

From the above information it can be concluded that in addition to the factors listed earlier (pH, temperature, etc.), which influence the resulting reduction of the acrylamide content in the final product, it is necessary to take into account the technological process used to prepare the specific product.

4. Methods for the determination of L-asparaginase activity

The described technological applications of L-ASNases cannot exist without the determination of the activity of enzyme preparations and also of the concentra-

tion of free L-asparagine. Different methods have been developed and described for measuring the activity of L-ASNase, and these methods mainly consist of determining the amount of the products in the reaction catalyzed by these enzymes, i.e. ammonia or L-aspartic acid[1]. The ammonia released during the asparaginase reaction can be determined by the Berthelot method, by reaction with Nessler's reagent, with indophenol[69] or can be detected with an ion-selective electrode[70]. The resulting L-aspartic acid can be determined, for example, by HPLC (ref.[71]), circular dichroism[72], or by reaction with hydroxylamine and ferric chloride[73]. Other methods for the determination of L-asparaginase activity use alternative substrates: 5-diazo-4-oxo-L-norvaline, L-aspartic acid β-(7-amido--4-methylcoumarin), and β-L-aspartic acid hydroxamate[74].

In the use of L-ASNases in medicine and food industry, not only the activity of the enzyme preparation is evaluated, but also the amount of unsplit L-asparagine after the application of the preparation, which determines its effectiveness and therefore its applicability. High-pressure chromatography is currently used to analyze the amount of L-asparagine present, but this is an instrumental and time-consuming method[75].

Finding a simple, fast, easily quantifiable, and sufficiently sensitive method, preferably using non-toxic compounds, would undoubtedly lead to the development of another area of application for L-ASNases, specifically in the design of biosensors[18,76]. A number of research teams are looking into the possibility of creating biosensors containing L-ASNase, which could be used to monitor blood levels of L-asparagine during cancer treatment[3]. At the same time, these biosensors could find similar applications in the food industry, where they could be used to rapidly verify the amount of the free L-asparagine in raw materials at risk of acrylamide formation during processing[3].

5. Conclusion

In this short overview article, we have tried to present and summarize the possibilities of using L-ASNase in the healthcare and food industry. As can be seen, these enzymes have a great potential for various fields, which have not yet been fully exploited. Therefore, current research is mainly focused on optimizing the recombinant production and purification parameters of these enzymes, as well as on characterizing L-ASNases from new sources that could provide enzymes with properties more favorable for medical or biotechnological applications. Modern genetic engineering techniques are not left behind, making it possible to modify the properties of already known enzymes artificially or to approximate their structure to human L-ASNase. These approaches could reduce the undesirable side effects arising from the foreign origin of the enzymes currently used.

Abbreviations

L-ASNase	L-asparaginase
ALL	acute lymphoblastic leukemia
GAS	"Group A Streptococcus"
EFSA	European Food Safety Authority
EMA	European Medicines Agency
FAO	Food and Agriculture Organisation
FDA	Food and Drug Administration
K_M	Michaelis constant
MHRA	Medicines and Healthcare Regulatory products Authority
PEG	polyethylene glycol
PMDA	Pharmaceuticals and Medical Devices Agency
WHO	World Health Organisation

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