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Deoxyribonuclease inhibitors

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ABSTRACT

Deoxyribonucleases (DNases) are a class of enzymes able to catalyze DNA hydrolysis. DNases play important roles in cell function, while DNase inhibitors control or modify their activities. This review focuses on DNase inhibitors. Some DNase inhibitors have been isolated from various natural sources, such as humans, animals (beef, calf, rabbit and rat), plants (*Nicotiana tabacum*), and microorganisms (some *Streptomyces* and *Adenovirus* species, *Micromonospora echinospora* and *Escherichia coli*), while others have been obtained by chemical synthesis. They differ in chemical structure (various proteins, nucleotides, anthracycline and aminoglycoside antibiotics, synthetic organic and inorganic compounds) and mechanism of action (forming complexes with DNases or DNA). Some of the inhibitors are specific toward only one type of DNase, while others are active towards two or more. Physico-chemical properties of DNase inhibitors are calculated using the Molinspiration tool and most of them meet all criteria for good solubility and permeability. DNase inhibitors may be used as pharmaceuticals for preventing, monitoring and treating various diseases.

1. Introduction

Deoxyribonucleases (DNases) are a heterogeneous class of enzymes which catalyze hydrolysis of deoxyribonucleic acid (DNA). Two main types of DNase are DNase I and DNase II, both endonucleases, which produce 3′-oligonucleotides and 5′-oligonucleotides, respectively [1,2]. DNases play an important role in pathogenesis of various diseases and programmed cell death (apoptosis) [3], while DNase inhibitors are compounds able to control or modify those activities [1]. Nucleosomal DNA fragmentation is the main characteristic of apoptosis in which are involved three main endonucleases: caspase-activated DNase (CAD), endonuclease G and DNase γ [4]. Among them, CAD is the major responsible nuclease in apoptotic DNA fragmentation [5].

Both DNases and their inhibitors may be used as diagnostic agents and for the monitoring and treatment of various diseases [6–8]. Serum DNase I activity may be a useful biochemical marker for early diagnosis of acute myocardial infarction and transient myocardial ischemia [6], while acid DNase activity may be a useful indicator of the ongoing apoptosis in reflux esophagitis [7]. Human pancreatic DNase I inhibitor is a reliable indicator of the pancreatic inflammation state and can be used for early detection of chronic pancreatic disorders [8]. It is possible that DNase γ inhibitors may become novel lead compounds for development of new pharmaceuticals for the treatment of sepsis, cerebral ischemia and other inflammatory diseases caused by HMGB1 release [9].

The aim of this article is to review the literature on natural and synthetic inhibitors of DNases. It will first focus on some of the main properties of different DNase types, followed by a description of natural and synthetic DNase inhibitors, including their chemical structure and mechanism of action. Further, physico-chemical properties of DNase inhibitors will be calculated. Finally, future perspectives for these compounds will be discussed. To the best of our knowledge, this is the first review of DNase inhibitors.

2. Properties of different types of DNases

2.1. DNase I family

The human DNase I family consists of four distinct DNases: DNase I, DNase X, DNase γ and DNAS1L2. DNase I, DNase X, and

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DNase γ are neutral endonucleases showing their maximum activity at neutral pH (6.8, 6.8 and 7.2, respectively), while DNAS1L2 is an acidic endonuclease showing its maximum activity at acidic pH (5.6). DNase I enzymes are Ca^{2+}/Mg^{2+}-dependent endonucleases. They are originally secretory proteins, but after acquisition of C-terminal domains, DNase γ and DNase X are retained intracellularly unlike DNase I or DNAS1L2 which are effectively secreted extracellularly [10]. Based on their chemical structure, all DNases I are glycoproteins [2]. Based on the different tissue distribution, there are three types of DNases I in mammals. The first one, present in human and pig, is termed pancreas-type due to the highest activity of DNase I in pancreas. This type is more sensitive to low pH values than other types of DNase I. The second, present in rat and mouse, is termed parotid-type due to the highest activity of DNase I in parotid glands. The third, present in bovine and rabbits, is termed pancreas-parotid or mixed type due to high activity of DNase I in both pancreas and parotid glands. Furthermore, DNases I are present in kidney, liver, stomach, small intestine, large intestine, spleen, heart, lung, cerebrum and cerebellum, but the activities in these tissues are moderate or very low [11].

Among the DNase I family, only DNase γ is able to cause apoptotic DNA fragmentation in mammalian cells. DNase γ is located in the perinuclear region in living (nonapoptotic) cells and is translocated into the nucleus during apoptosis [10]. The nickase activity of DNase γ depends on several factors, such as ionic strength, protein cofactors and DNA structures. DNase γ is a Mg^{2+}/Ca^{2+}-dependent single-stranded DNA nickase with high activity at low ionic strength, while under higher (physiological) ionic strength it exhibits endonuclease activity in the presence of a coactivator histone H1 [4].

2.2. DNase II family

DNase II family consists of highly homologous DNases with ubiquitous tissue distribution, lysosomal localization and optimal function at acid pH without divalent cations. DNase II enzymes play important roles in engulfment-mediated DNA degradation as the main mechanism of DNA removal which is critical for proper development and homeostasis. Deficiency in DNA clearance, associated with mutations in DNase II enzymes, may lead to some autoimmune dysfunctions, such as systemic lupus erythematosis [12]. L-DNase II is an enzyme involved in apoptosis, derived from porcine leucocyte elastase inhibitor (LEI), a protein from the serpin superfamily. In living cells, LEI is in its native form and has no effect on DNA degradation due to its anti-protease activity. During apoptosis, reduced intracellular pH induces posttranslational modification of LEI which involves a shift in the molecular weight of LEI followed by a loss of its anti-protease activity and the appearance of L-DNase II. This leads to the activation of two degradation pathways involved in apoptosis: protease and nuclease pathways [13].

2.3. Caspase-activated DNase

DNA fragmentation factor (DFF) is a heterodimeric protein consisting of two subunits, DFF40 (~40 kDa), also named caspase-activated DNase (CAD), and DFF45 (~45 kDa), also named the inhibitor of caspase-activated DNase (ICAD). DFF40/CAD is the active component of the complex which causes both DNA fragmentation and chromatin condensation during apoptosis [14,15]. Among examined apoptotic proteinases, caspases-3, -6, -7, -8 and granzyme B cause apoptotic DNA fragmentation via inactivation of DFF45/ICAD and release of active DFF40/CAD. Caspase-3 and caspase-7 promote DFF45/ICAD inactivation and DNA fragmentation directly by benzoyloxycarbonyl-Asp-Glu-Val-Asp (DEVD) cleavage with caspase-3 being more effective than caspase-7. On the other hand, granzyme B, caspase-6 and caspase-8 promote DNA fragmentation indirectly by activation of caspase-3 or caspase-7. Therefore, caspase-3 has the primary role in DFF45/ICAD inactivation and apoptotic internucleosomal DNA fragmentation [16]. Granzyme M (GzmM) is a protease that causes direct degradation of ICAD resulting in CAD release and DNA fragmentation (not DNA nicking) during apoptosis. GzmM also forces apoptosis by cleavage of poly(ADP-ribose) polymerase responsible for DNA repair [17]. Granzyme A (Gzm A) causes DNA nicking during apoptosis by cleavage of SET which is specific inhibitor of GzmA-activated DNase (GAAD) [18].

2.4. Drosophila melanogaster embryonic DNases

Three *D. melanogaster* embryonic DNases, named den1, den2 and den3, were isolated from nuclear extracts. Den1 exhibits 5′-exonucleolytic activity toward both single-stranded and double-stranded DNA. Den2 exhibits 3′-exonucleolytic activity toward double-stranded DNA, but not toward single-stranded DNA. Den3 requires partial duplex DNA that contains single-stranded gap and it catalyzes DNA hydrolysis in 3′-5′ direction. The common property of den1, den2 and den3 is that their nucleolytic activities can be inhibited with ATP [19].

3. Natural DNase inhibitors

3.1. Natural DNase I inhibitors

Natural DNase I inhibitors have been isolated from various sources:

- humans: somatostatin [20], cholesterol sulfate in concert with bile acids [21], inhibitor in human leucocytes [22,23], and protein from KB cells [24];
- animals: actin [1,25,26], anti-DNase antisera [27,28], proteins from calf spleen [29], and thymus [30];
- microorganisms: antibiotics isolated from bacteria of the genus *Streptomyces* (actinomycin D, nogalamycin, daunomycin, neomycin B and paromomycin) [31,32], and a metabolite from *Micromonospora echinospora* [33];
- plants: protein from *Nicotiana tabacum* cells [34];

Somatostatin is the first physiological factor able to regulate DNase I activity *in vivo*. Intraperitoneal administration of somatostatin to rats results in decreased level of serum DNase I in a dose-dependent manner. Reduction of DNase I activity is due to down regulation of DNase I gene expression. Somatostatin decreases levels of DNase I activity in pituitary gland, stomach, small intestine, colon and serum, but does not change enzyme activity in the parotid gland, liver and kidney. Since the tissue origin of rat serum DNase I is unknown, serum enzyme levels are used to reflect those in the lower gut and pituitary gland. Based on different response to somatostatin, rat tissues expressing DNase I can be classified into two types: somatostatin-sensitive (the body of the stomach, duodenum, jejunum, ileum and colon) and somatostatin-resistant (esophagus and fundus of the stomach) [20].

Gastric fluids contain cholesterol sulfate (CS) at concentrations of 14–131 μg/mg of protein. CS alone shows no inhibitory effect toward DNase I even at 50 μg, but completely abolishes DNase I activity when it is solubilized with DMSO or bile acids in phosphate buffer. The inhibition is irreversible and dose-dependent. The sulfate group and the hydrophobic side chain of CS are both required for the inhibitory effect. Also, an optimal molar ratio of bile acids to CS is required (0.18 for sodium taurocholate to CS). Lower molar

ratios disable formation of micelles of CS, and higher molar ratios prevent the interaction of CS with DNase I. CS is a specific inhibitor for pancreatic DNase I because other lipid components in the digestive tract, such as cholesterol, sulfatides, free fatty acids, triglycerides, phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine, sphingomyelin and galactosylceramide do not inhibit DNase I [21].

An inhibitor for human serum DNase I is present in human leucocytes. It is a protein, soluble in saline, stable at 56 °C and species-specific to some extent. The only exception is rabbit where the inhibitor is equally effective as in human [22]. Addition of 0.5 ml leucocyte extract to the incubation mixture is able to inhibit human serum DNase I in a competitive manner [23].

A protein from KB cells with a molecular weight of approximately 140 kDa is able to inhibit DNA hydrolysis by KB DNase I. The inhibitor binds to the DNA substrate and has the effect only on the hydrolysis of single-stranded, but not double-stranded, DNA [24].

Actin, one of the major structural proteins of muscle and non-muscle cells, is a widely occurring inhibitor of DNase I. It was observed that skeletal muscle actin has an amino-acid composition similar to naturally occurring specific inhibitors of DNase I. Actin exhibits inhibitory activity toward DNase I by forming a stable complex with the enzyme [1]. Only the monomeric form of actin inhibits DNase I, but not filamentous form [35]. The physiological function of the DNase I-actin interaction is to protect cells from premature chromatin degradation and cell death [25]. Lacks [26] examined inhibition of DNase I from various sources by rabbit muscle actin and came to the conclusion that actin inhibited the bovine parotid and pancreatic DNase I, but did not inhibit any of the rat DNase I. The results of that study indicate that actin inhibition is species-specific. On the other hand, Mannherz et al. [36] showed that DNase I activity in both rat and bovine parotid tissue extracts can be inhibited by monomeric actin. They gave an explanation for the discrepancy with Lacks' observations [26]. The amount of DNase I activity is different in rat and bovine parotid gland and pancreas. There is also a difference in their molecular weight and in their affinity for monomeric actin (being lowest for rat parotid DNase I (5×10^6 M^{-1})). Rat parotid DNase I is unable to depolymerize filamentous actin (F-actin). After prolonged incubation of parotid gland tissue extracts, the inhibitory action is reversed due to the simultaneous presence of 5′-nucleotidase in rat parotid extract, especially when low concentrations of actin are used. Using a technique of enzyme detection after gel electrophoresis in the presence of sodium dodecyl sulphate (SDS), it is possible to change the actin-binding ability of DNase I in a species-specific mode. Difference in the resistance to low pH between rat and bovine DNase I was also observed [36].

Abe et al. [27] reported that rabbit antisera inhibit DNase I from bovine pancreas and parotid and from ovine pancreas, while porcine pancreatic and malted barley DNases remain active. Inactive DNase I, carboxymethylated at the active site His^{134} (CM-His134-DNase), reverses the antisera inhibition, suggesting that the epitope for antisera binding does not contain His^{134}. The antibody, but not the other serum factors in the anti-DNase antisera, exhibit inhibitory effect due to antibody binding to the 153–163 region of DNase I and prevent the binding of Ca^{2+} or other metal ions at site I, the modulating site of DNase I. The 153–163 region contains three peptide bonds, Lys-Trp-His-Leu and it is not crucial for enzymatic catalysis, but is required for double-strand cuts. The degree of DNase I inhibition depends on the metal ion used as DNase activators. With Mn^{2+}, Co^{2+} or Mg^{2+} plus Ca^{2+} the inhibition is about 50%, while with Mg^{2+} alone the inhibition is insignificant [28].

Lindberg [29] reported isolation of two proteins from calf spleen that show inhibitory effect toward DNase I and defined them as inhibitor I and inhibitor II. Both inhibitors I and II form stable complexes with DNase I with a 1:1 stoichiometry [29]. Inhibitor II is a more homogeneous protein with an average molecular weight of 59.4 kDa [37] and displays higher specific activity toward DNase I than inhibitor I [29]. The composition of inhibitor II obtained by amino acid analysis is $Asp_{49}Thr_{38}Ser_{35}Glu_{54}Pro_{27}Gly_{44}Ala_{42}$-$Val_{37}Met_{20}Ile_{34}Leu_{42}$-$Tyr_{19}Phe_{19}Lys_{31}His_{12}Arg_{25}$(total half-$cys_9$)$(CONH_2)_{28}$. It is noticeable that the content of sulfur-containing and aromatic amino acids is relatively low [37]. Treatment with acidic pH (3.5), urea (3–5 M), or *p*-hydroxymercuribenzoate results in aggregation and inactivation of the inhibitor II [29]. It is a labile protein, but can be stabilized with potassium phosphate buffer of high ionic strength or by the addition of glycine at high concentration [37]. A protein isolated from calf thymus inhibits pancreatic DNase I and has some identical properties as spleen inhibitor II. They both form high molecular weight aggregates, both are specific for DNase I and form complexes with a 1:1 stoichiometry. On the other hand, optimal stability is achieved in different conditions due to the different structure of these two proteins. Also, the molecular weight of the thymus inhibitor (48.7 kDa) is lower than that of the spleen inhibitor II (59 kDa) [30].

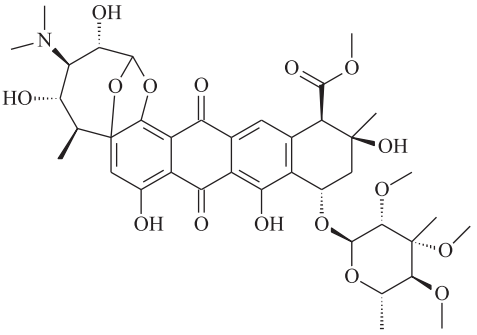
Nogalamycin, daunomycin and actinomycin D (Fig. 1) are antibiotics able to inhibit DNase I activity by forming stable complexes with DNA. They displace methyl green (MG) from its DNA complex and the degree of displacement depends on the percentage of adenine and thymine in DNA. Nogalamycin and daunomycin are better inhibitors of DNase I and displace more MG than actinomycin D. They are both anthracycline antibiotics and therefore show similar activity. On the other hand, actinomycin D is a polypeptide antibiotic that acts differently than nogalamycin and daunomycin, and does not completely displace MG [31]. Among aminoglycoside antibiotics, neomycin B (Fig. 1) is the most effective against DNA degradation. Neomycin molecules strongly interact with DNA and therefore inhibit DNase I binding. Neomycin B completely inhibits degradation of plasmid DNA *in vitro* at a concentration of 2 mM. Paromomycin (Fig. 1) has also protective effects against DNA degradation but only at increased concentrations because of additional hydroxyl group (instead of a positively charged amino group in neomycin) which cause unfavorable electrostatic interactions and lower affinity to the phosphate backbone [32]. It has been found that the low molecular weight compound from *M. echinospora* MG299-fF35 exhibits inhibitory activity toward DNase I with IC_{50} values of 6.1 μg. This compound inhibits DNase I activity by direct binding to the enzyme but the structure was not determined [33].

The first specific inhibitor of DNase I isolated from plants was extracted from *N. tabacum* cell cultures. It is a homogeneous protein with a molecular weight of 14.5 kDa. This protein reduces the hydrolysis of *Escherichia coli* DNA by DNase I and also reduces the activity of DNA specific nuclease isolated from tobacco cell cultures. However, it is inactive toward micrococcal nuclease suggesting that this protein inhibitor is specific for the active site of eucaryotic DNases. The inhibitor protein interacts with DNase I in the absence of DNA and forms a DNase-inhibitor complex in a 1:1 stoichiometric ratio. This interaction shows strong temperature dependence with an average dissociation constant of 5.2 nM at 20 °C and 110 nM at 26 °C [34].

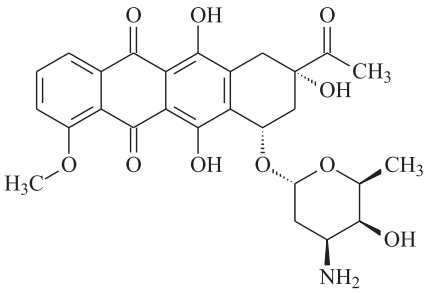
3.2. Natural DNase II inhibitors

It was found that proteins from animal tissues [38–40] and KB cells [24], some nucleotide compounds [41] and microorganism metabolites [33] exhibit inhibitory activity toward DNase II.

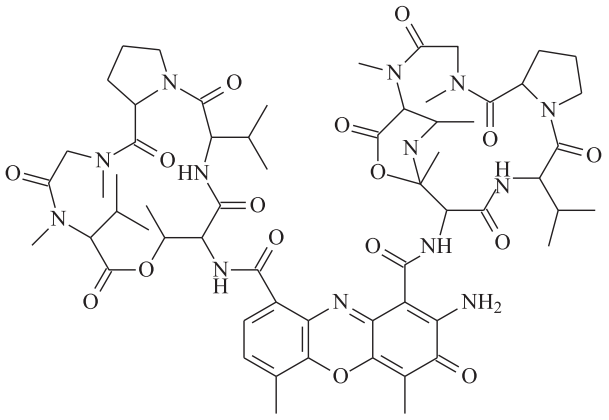
A protein isolated from mouse liver is able to inhibit beef spleen and *Helix pomatia* acid DNases, but not neutral DNases, pancreatic DNase and *E. coli* endonuclease I. The enzyme kinetics are



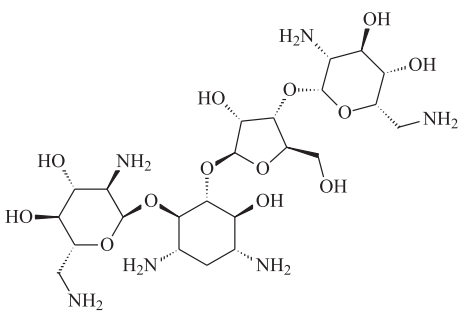
Nogalamycin



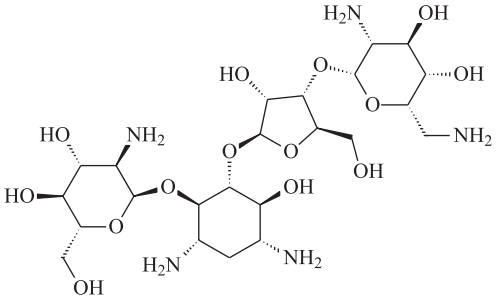
Daunomycin



Actinomycin D



Neomycin



Paromomycin

Fig. 1. Chemical structures of natural DNase I inhibitors.

Michaelis–Menten type in the absence of inhibitor, while in its presence the kinetics become sigmoidal. The effect is more pronounced with higher inhibitor concentrations. With higher substrate concentrations inhibitory effect becomes weaker, while it is completely lost at its highest concentration (35 μM). The DNase-inhibitor interaction is very sensitive to pH changes – inhibition disappears after just a small pH shift from 5.1 to 5.57. This inhibitor is able to reactivate the urea-treated DNase by interacting with two inactive monomers and reassociating them to an active dimeric form. The DNase-inhibitor complex consists of two subunits: the catalytic (active enzyme) and regulatory subunit (inhibitor) [38]. A protein isolated from beef liver exhibits inhibitory activity toward acid DNase and forms an enzyme-inhibitor complex with a 1:1 stoichiometry. The molecular weight of this inhibitor is 21.5 kDa. Its maximum activity is at pH 5.0, while the enzyme-inhibitor interaction disappears with a small pH shift from 5.0 to 5.57. The enzyme kinetics are Michaelis–Menten type in the absence of the inhibitor, while in its presence the kinetics become sigmoidal. The sigmoidal shape is more pronounced with higher concentration of the inhibitor. In addition, RNA which binds to the catalytic site and inhibits acid DNase is able to reverse the binding of inhibitor to DNase [39]. Bovine serum albumin (BSA) is able to inhibit acid DNase from rat small intestinal mucosa. This inhibition is pH-dependent: BSA has a maximum inhibitory effect at pH 4.3, while at pH 4.7 it exhibits a contradictory stimulative effect. The stimulative effect is heat-labile due to heat-inactivation at all pH values,

while the inhibitory effect is heat-stabile due to heat-inactivation at pH 5, but not at lower or higher pH (pH 4.3 or 6.0). After cyanogen bromide-cleavage of BSA, two fragments can be obtained: fragment C with an inhibitory effect nearly equal to that of the intact BSA, and fragment N, without inhibitory activity. Reduction of fragment C leads to decreased inhibitory effect, while the activity is completely lost after its separation into three component peptides. Also, the inhibitory activity of BSA is completely lost after its acetylation. Thus, the three-dimensional structure of the albumin is required for the inhibitory effect. This inhibition is not species-specific because acid DNases from various species can be inhibited by serum albumin in the same way as rat small intestinal acid DNase by BSA [40].

Natural nucleotide compounds, such as s-RNA (yeast soluble ribonucleic acid) and rRNA (ribosomal RNA) from Ehrlich ascites tumor cells are able to inhibit hog spleen acid DNase activity in a competitive manner [41]. Protein from KB cells and the low molecular weight compound from *M. echinospora* MG299-fF35, previously described in chapter 3.1, exhibit inhibitory activity toward KB DNase II [24] and DNase II (IC_{50} is 9 μg) [33], respectively.

3.3. Natural DFF40/CAD inhibitors

Some naturally occurring compounds, such as DFF45/ICAD [14,15], DFF35/$ICAD_S$ (DNA fragmentation factor 35/inhibitor of caspase-activated DNase (short form)) [42], Akt/Ebp1 complex [43] and natural pigment curcumin [44], are able to inhibit DFF40/CAD and thus prevent DNA fragmentation during apoptosis. Inhibitors homologous to ICAD have been identified in *Xenopus laevis* egg extract (IXAD) [45] and *D. melanogaster* S2 cells (DREP-1/dICAD) [46].

DFF45/ICAD is a specific inhibitor of DFF40/CAD. There are reactive histidine residues within the active site of CAD that are essential for the enzyme activity. ICAD blocks the DNA binding to CAD by steric or electrostatic hindrance, but has no influence on chemical modification of the histidine residues, suggesting that histidine residues (and so the active site of CAD) are not involved in this binding [47]. The main target for ICAD is the dimerization (C2) domain of CAD, but it also interacts with the Zn^{2+}-binding site and the catalytic center in the C3 domain of CAD. Two ICAD-derived peptides (B1 and B2) are able to efficiently inhibit CAD [15]. There are short (ICAD-S) and long (ICAD-L) splice forms of the ICAD protein in cells. ICAD-S acts as an inhibitor and reserves as a buffer to prevent inappropriate CAD activation, but is unable to function as a chaperone for CAD. ICAD-L acts both as activator and inhibitor of CAD and functions as a chaperone to promote the folding of active CAD, but does not act as a buffer [48]. DFF35/$ICAD_S$ is the prevalent form of ICAD expressed in the rodent brain cells. This protein forms a functional complex with DFF40/CAD in the nucleus and inhibits apoptotic DNA fragmentation. During neuronal apoptosis, caspases translocate to the nucleus, inactivate DFF35/$ICAD_S$, bind with DFF40/CAD and activate DFF40/CAD. Thus, DFF35/$ICAD_S$ is the endogenous DFF40/CAD inhibitor in neurons [42].

Ahn et al. [43] showed that the Ebp1 protein is responsible for preventing DNA fragmentation during apoptosis. Because of high Ebp1 amounts required to express the inhibitory effect under physiological conditions, phosphorylation or binding partners might be needed. Ebp1 phosphorylated by protein kinase C interacts with phosphorylated nuclear, but not cytoplasmic, Akt and suppresses apoptosis. The antiapoptotic action of Akt is independent of its kinase activity. Hence, active nuclear Akt/Ebp1 complex inhibits DFF40/CAD and prevents DNA fragmentation during apoptosis [43].

The natural pigment curcumin (Fig. 2) is able to induce caspase-3 activity leading to DFF45/ICAD inactivation and release of DFF40/CAD, but it does not cause oligonucleosomal DNA fragmentation. That is because curcumin inhibits DFF40/CAD endonuclease activity via direct binding and thus blocks the active site of DFF40/CAD. The effect of curcumin depends on the cell type and concentration. It protects human T cells against apoptotic DNA fragmentation, but does not prevent cell death [44].

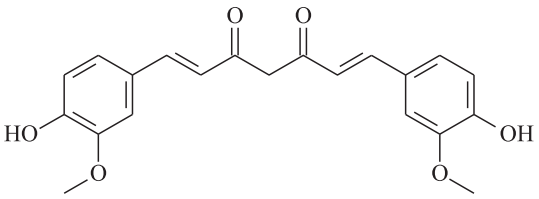


Fig. 2. Chemical structure of curcumin.

An apoptosis-specific DNase, similar to CAD, was identified in *X. laevis* egg extract *X*S-150 and termed XAD, while its specific inhibitor is IXAD. IXAD is a protein that normally exists in *X. laevis* egg extract in two forms: in the form of dimer or in complex with XAD. During apoptosis, IXAD is cleaved, leading to the release and activation of XAD and consequently to DNA fragmentation. This protein is likely homologous to DFF45 in both structure and function based on Western blot and cross-inhibition studies [45]. Mukae et al. [46] identified DREP-1/dICAD in *D. melanogaster* S2 cells, inhibitor homologous to mouse or human ICAD (about 17% of identity). Despite this low percentage of homology, DREP-1/dICAD meets the criteria for classification as ICAD. It forms a complex with *Drosophila* CAD (dCAD), inhibits dCAD activity and thus prevents the apoptotic DNA fragmentation. It can be cleaved at a specific site by human caspase-3 resulting in the loss of its inhibitory activity. However, *Drosophila* S2 cells express only ICAD-L, but not the ICAD-S form of ICAD [46].

3.4. Other natural DNase inhibitors

Various compounds from microorganisms show inhibitory effect toward DNases [33,49,50]. Further, infection of *Salmonella typhimurium* with phage P22 inhibits DNase activity [51]. Some vitamins are also able to affect DNase activity [52,53].

E. coli DNA binding protein does not inhibit the formation of partial or whole single-stranded fragments from linear duplex DNA catalyzed by *recBC* DNase, but it does inhibit hydrolysis of this material to acid-soluble oligonucleotides. This protein also inhibits hydrolysis of duplex circular DNA containing short, single-stranded gaps [49]. Infection of KB cells by adenovirus type 5 (Ad5) leads to decreased levels of cellular DNase activity because of adenovirus-specific DNA binding protein which acts as DNase inhibitor and binds to the single-stranded DNA. On the other hand, a DNA-negative mutant Ad5 *ts*125 is unable to inhibit DNase activity because of its failure to induce DNA binding protein. However, inhibition of DNase activity occurs after infection by DNA-negative mutant Ad5 *ts*36 complement to *ts*125. Since protein synthesis is required for the decreased levels of DNase activity after adenovirus infection, this decrease can be prevented by the inhibition of protein synthesis by cycloheximide [50]. The low molecular weight compound from *M. echinospora* MG299-fF35, previously described in chapter 3.1, exhibits inhibitory activity toward DNases from *Streptomyces* strains 6016, 4290, 4098 [54] with IC_{50} values of 9.7, 42 and 47 μg, respectively. This compound inhibits DNase activity by direct binding to the enzyme [33]. Infection of *S. typhimurium* with phage P22 leads to the inhibition of DNase activity toward single-stranded DNA. However, there is no inhibition after infection by phage with a mutation in gene 25 (*P22ts25.1*) or if the host cell carries a P22 prophage, but it does occur after infection by other mutants (*ts*l2 and *ts*l8) which are defective in phage DNA synthesis.

The inhibition of DNase is reversible *in vivo* if the invading phage is wild type *P22c+*, while *in vitro* it can be reversed by dialysis. By inhibiting protein synthesis, chloramphenicol can prevent the decrease of DNase activity in infected cells, suggesting that some phage-coded protein is synthesized which causes reversible DNase inhibition [51].

Some vitamins can also affect the activity of DNases. Vitamin C and vitamin K3 act as DNase activators and may be considered as a possible new, non-toxic, adjuvant cancer therapy [52]. On the other hand, two forms of vitamin B6, pyridoxal 5′-phosphate and pyridoxal (Fig. 3), exhibit inhibitory activity towards DNase [53]. Pyridoxal 5′-phosphate and pyridoxal are able to inhibit *Bacillus laterosporus* ATP-dependent DNase. The inhibition can be reversed by dilution or dialysis, while $NaBH_4$ inhibition is irreversible due to reduction of the enzyme-pyridoxal 5′-phosphate complex. However, $NaBH_4$ has no influence on DNase inhibition when it is used alone. *B. laterosporus* DNase is a specific enzyme which has two different independent binding sites: a DNA binding site and an ATP binding site. Inhibition is competitive with DNA as substrate, while ATP has no effect. Pyridoxal 5′-phosphate forms Schiff base with ε-NH_2 group of an essential lysine residue at the DNA binding site. The aldehyde group of pyridoxal 5′-phosphate is essential for effective inhibition, while the phosphate group enhances the inhibition [53].

4. Synthetic DNase inhibitors

4.1. Synthetic DNase I inhibitors

Some derivatives of 2-nitro-5-thiobenzoic acid [55,56], hydroxybiphenyls [57], nitrogen mustard [23], crystal violet [58] and some anionic polyelectrolytes [59,60] were found to act as DNase I inhibitors.

2-Nitro-5-thiocyanobenzoic acid (NTCB) and 2-nitro-5-thiosulfobenzoic acid (NTSB) (Fig. 4) are thiol-specific modifying compounds able to inhibit DNase I activity in the presence of Ca^{2+}/Mg^{2+} at pH 7.5 [55,56]. NTCB-inactivation of DNase I is irreversible and is a result of covalent modification of the enzyme. NTCB causes not only inactivation but also fragmentation of the enzyme to several minor low molecular weight fragments. However, one part of the inactivated enzyme remains uncleaved. During the reaction between NTCB and DNase, hydrogen of DNase is displaced with the cyano group of NTCB forming cyano-DNase and TNB (2-nitro-5-thiobenzoic acid) [55]. On the other hand, NTSB-inactivation of DNase I is reversible by acid treatment when inactivated enzyme regains about 40% of its initial activity. Inactivation is also through covalent modification, but without fragmentation of the polypeptide chain [56]. Amino acid analyses show that both completely inactivated and the native enzymes contain all four half-cystine (cysteine with the hydrogen atom removed from the thiol group) residues suggesting that reaction does not occur at the half-cystine residues. Possible reaction sites for NTCB and NTSB may be threonine and serine because of their reduced values during analyses. NTCB and NTSB do not significantly inactivate α-chymotrypsin, egg white lysozyme, snake venom phosphodiesterase, bovine pancreatic ribonuclease and bovine spleen acid DNase. Therefore, NTCB and NTSB are unique inhibitors of DNase I but not all DNases [55,56].

Mono- and dihydroxybiphenyls can harm DNA hydrolysis by DNase I by forming complexes with DNA substrate rather than directly affect DNase I. Using molecular models of DNA and several hydroxybiphenyls, Gottesfeld et al. [57] found that the interaction between hydroxybiphenyls and DNA is based on the intercalation of the unsubstituted phenyl group between the nucleic acid bases, and on the hydrogen bonding of the hydroxyls on the other phenyl group with the nucleic acid bases. Thus, it is required for hydroxybiphenyls to have free hydroxyl group and an unsubstituted (or *o*-substituted) phenyl group to express inhibitory activity. Some bulky groups may harm inhibition by preventing intercalation because of steric hindrance. Certain intramolecular position of the hydroxyl group and π-cloud interactions between phenyl groups and nucleic acid bases are also required. Hydroxyl group at the *m*-position is the most effective and can easily form hydrogen bonds with the nucleic acid bases. Interaction is more difficult when hydroxyl group is at the *o*-position, and quite impossible when the hydroxyl group is at the *p*-position of the phenyl group [57].

Chemotherapeutic drug nitrogen mustard (methyl-bis(β-chloroethyl)amine hydrochloride) (Fig. 4) is able to inhibit the activity of human serum DNase I in a noncompetitive manner when 2 mg is added to the incubation mixture [23]. Triphenylmethane dye crystal violet (Fig. 4) is able to inhibit DNase I activity with an IC_{50} value of 0.35 μM [58].

Anionic polyelectrolyte, hydrolyzed ethylene maleic anhydride (HEMA), has no inhibitory effect on DNase I at its optimum pH (pH 7.5), but shows moderate effects on DNase I at a pH lower than optimum (pH 5.0). However, much larger quantities of polymer (100 μg) are required for just 50% inhibition of DNase I. HEMA does not inhibit DNase I at pH 6 and above [59]. Poly ethenesulfonic acid (PES) stimulates DNase I activity at intermediate concentrations, while at higher concentrations it inhibits DNase I. These results are contrary to those of Tunis and Regelson [59] who did not observe any effect of PES on DNase I [60]. Bach [60] considers that this discrepancy is a result of different compound concentrations, different assay methods and different composition of the incubation mixture.

Oligonucleotide d[ApAp(S)ApA], the phosphorothioate analog of the d[ApApApA] tetramer, used as a mixture of the Rp- and Sp-diastereomers, is resistant to hydrolysis catalyzed by DNase I. Since the phosphorothioate group of either diastereomer is not hydrolyzed, it means that incorporation of phosphorothioate groups into oligonucleotides and DNA can provide protection against enzymatic cleavage [61].

4.2. Synthetic DNase γ inhibitors

Various triazine derivatives show inhibitory effects toward DNase γ [62,63]. Sunaga et al. [62] screened inhibitors of apoptotic DNA fragmentation from their chemical library and found a novel

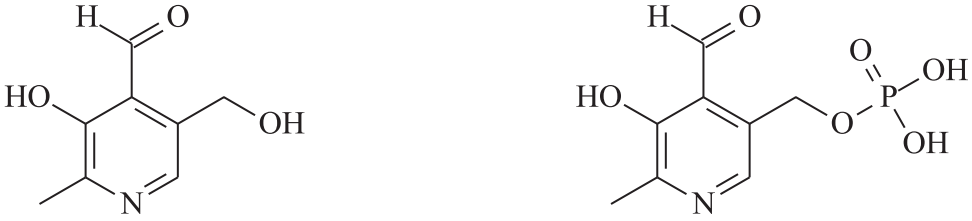
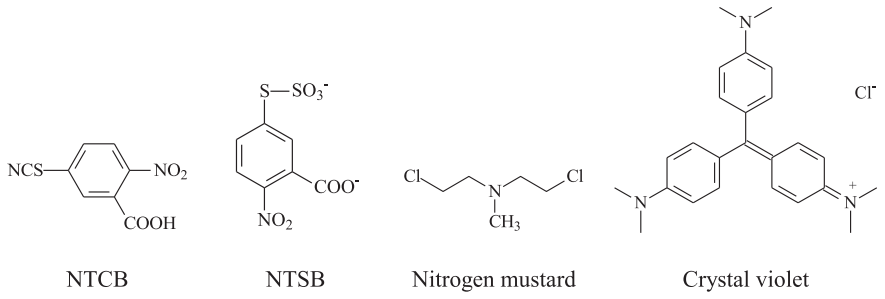


Fig. 3. Chemical structures of pyridoxal and pyridoxal 5′-phosphate.



NTCB NTSB Nitrogen mustard Crystal violet

Fig. 4. Chemical structures of synthetic DNase I inhibitors.

compound named DR396 (4-(4,6-dichloro-[1,3,5]-triazin-2-ylamino)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-benzoic acid) (Fig. 5). It is a potent and specific DNase γ inhibitor which inhibitory effect on DNase γ was examined in HeLa S3 cells (HeLa-γ cells) and rat splenocytes. Apoptotic DNA fragmentation was induced by staurosporine (STS) in HeLa-γ cells, and by γ-irradiation in rat splenocytes. DR396 directly inhibits DNase γ in a dose-dependent manner, without modulation of any other apoptotic processes. The compound does not delay apoptotic processes and has no effect on apoptotic DNA fragmentation catalyzed by CAD. Furthermore,

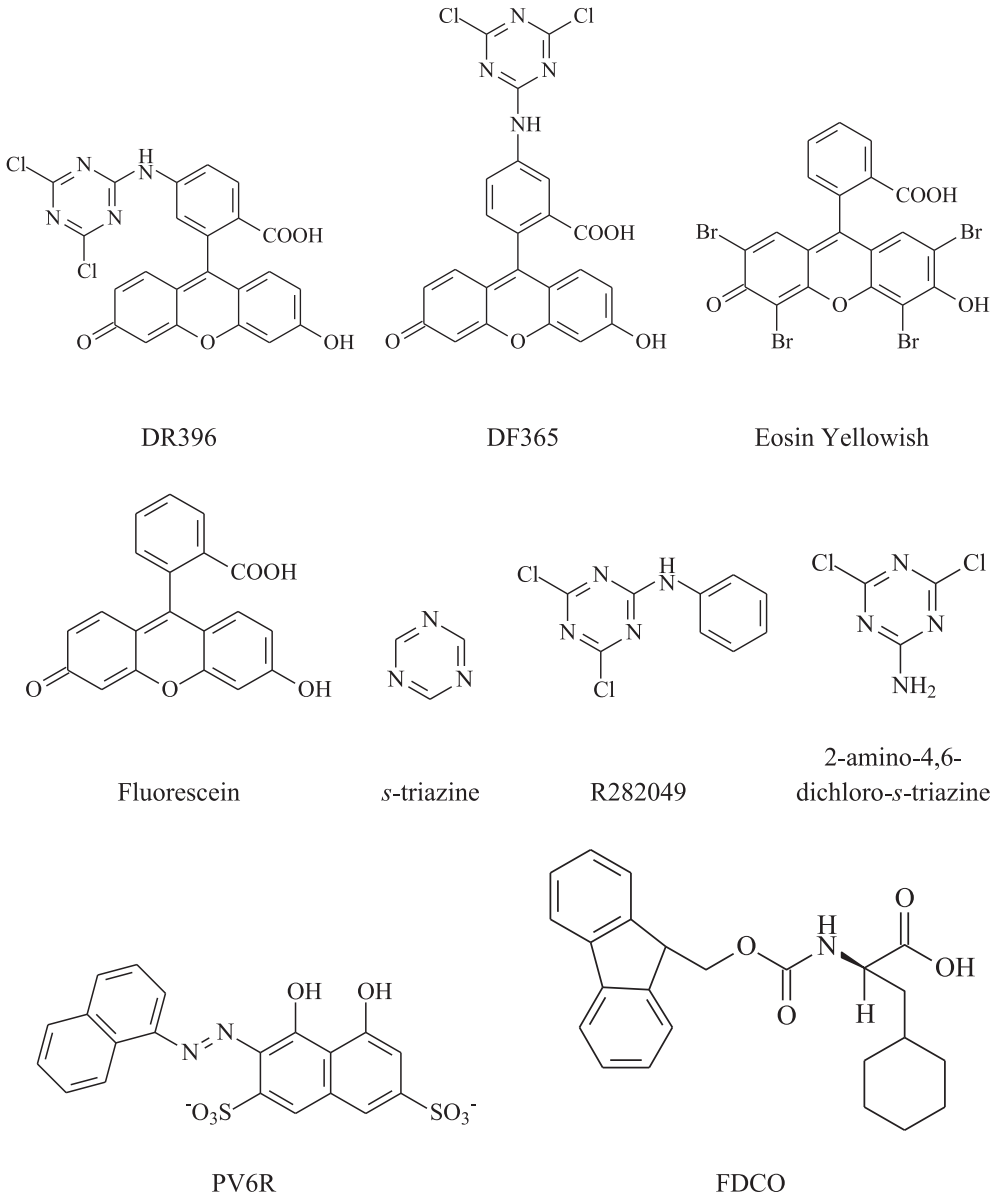


Fig. 5. Chemical structures of synthetic DNase γ inhibitors.

DR396 is about 100-fold more selective to DNase γ ($IC_{50} = 3.2$ μM) than DNase I ($IC_{50} > 300$ μM) [62]. The unique binding site of DR396 on DNase γ is a 'DNA trapping site' and it is not the active site. DR396 forms six hydrogen bonds with amino acid residues in the subsites S1, S2 and S3 of 'DNA trapping site'. Sunaga et al. [63] searched for other new DNase γ inhibitors by screening their chemical library of about 100,000 diverse compounds. They performed docking simulations with the 'DNA trapping site' of DNase γ and found some inhibitors with predicted high affinities for the 'DNA trapping site' (Fig. 5). One of them is DF365 (5-(4,6-dichloro-[1,3,5]-triazine-2-ylamino)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-benzoic acid), a regioisomer of DR396, but it is over 20-fold weaker inhibitor than DR396. DF365 does not form hydrogen bonds with the S3 subsite and the location of the triazine group is different compared to DR396. Eosin Yellowish is another analog of DR396. Partial structures of DR396, fluorescein and *s*-triazine, have lower affinities for the 'DNA trapping site' and lower inhibitory activity for DNase γ. While fluorescein binds to the S1 and S2 subsites, *s*-triazine binds only to the S3 subsite. Other derivatives of DR396 are (4,6-dichloro-[1,3,5]-triazine-2-yl)-phenyl amine (R282049) and 2-amino-4,6-dichloro-*s*-triazine, but they exhibit low inhibitory activity toward DNase γ [63].

It is known that high mobility group box1 (HMGB1) is secreted into extracellular space during apoptosis and necrosis and causes inflammatory responses. Some compounds which are known as DNase γ inhibitors are also able to inhibit HMGB1 release from apoptotic cells. That means that nucleosomal DNA fragmentation catalyzed by DNase γ plays an important role in HMGB1 release during apoptosis. Using staurosporine-induced apoptotic HeLa S3 cells, three compounds were screened: DR396, Pontacyl Violet 6R (PV6R) and Fmoc-D-Cha-OH (FDCO) (Fig. 5). DR396 is the most potent DNase γ inhibitor and almost completely inhibits HMGB1 release, while PV6R and FDCO are less effective. PV6R inhibits approximately 50%, while FDCO inhibits 20% of HMGB1 release [9].

4.3. Synthetic DNase II inhibitors

Some anionic polyelectrolytes (PES, HEMA and ammoniated ethylene maleic anhydride (AEMA)) in an amount of 10 μg are able to completely inhibit DNase II activity at its optimum pH values (pH 5.0) equally either in the presence or in the absence of Mg^{2+}. HEMA, with two free carboxyl groups in the repeating unit, has the maximum inhibitory effect with the smallest quantities. AEMA, with one free and one masked carboxyl group, is a less effective inhibitor (Fig. 6). And when both carboxyl groups are blocked, such as diamide, the inhibitory activity toward DNase II is completely lost. Inhibition of DNase II is reversible by increasing the salt concentration (NaCl) [59].

The activity of acid DNase is affected by the nitric oxide (NO) donor sodium nitroprusside (SNP) and peroxynitrite, which are known as promoters of apoptosis in different cell types. Kocic et al. [64] investigated if there was a different effect on DNase activity after *in vivo* and *in vitro* treatment with SNP or peroxynitrite. Both *in vivo* administration of SNP or peroxynitrite and *in vitro* treatment of isolated hepatocytes or purified enzymes with SNP or peroxynitrite resulted in reduced activity of acid DNase in a dose-dependent manner. The inhibition of DNase II may be a result of nitrosylation of its active site [64].

Table 1
The inhibition of DNase I by various inorganic substances.

Compound	Concentration	Inhibition
Sodium arsenate	0.1 M	Complete
Sodium selenite	0.1 M	Complete
Copper sulphate	0.01 M	Complete
Zinc sulphate	0.01 M	Complete
Sodium sulphide	0.125 M	Complete
Potassium fluoride	0.1 M	Complete
Sodium citrate	0.1 M	Complete
Sodium borate	0.1 M	Complete
Potassium fluoride	0.01 M	Partial
Sodium citrate	0.01 M	Partial
Thioglycolic acid	0.5 M	Partial

Synthetic nucleotide compounds, such as poly U (polyuridylic acid), poly I (polyinosinic acid) and the poly A-poly U complex (polyadenylic acid-polyuridylic acid complex) exhibit inhibitory activity toward hog spleen acid DNase. On the other hand, poly A and poly C (polycytidylic acid) have no effect on the DNase activity. The inhibition is competitive with poly A-poly U complex showing the strongest inhibitory effect [41]. Oligonucleotide d[ApAp(S)ApA], previously described in chapter 4.1, is also resistant to hydrolysis catalyzed by DNase II due to the incorporation of phosphorothioate groups [61].

4.4. Synthetic DFF40/CAD inhibitors

The activity of CAD may be affected by SNP and peroxynitrite which are previously described in chapter 4.3. Different effects on DNase activity are observed after *in vivo* and *in vitro* treatment with SNP or peroxynitrite. After *in vivo* administration of either SNP or peroxynitrite, the activity of CAD increases within 24 h. *In vitro* treatment of isolated hepatocytes or purified enzymes with either SNP or peroxynitrite results in reduced activity of CAD in a dose-dependent manner. *In vivo* administrated SNP and peroxynitrite are also able to induce DNA modifications, such as nitroguanine formation, tending to increase the rate of DNA fragmentation but not in a statistically significant manner [64].

5. Inorganic DNase inhibitors

Gilbert et al. [65] examined the inhibitory activity of some inorganic substances toward DNase I and found that some of them almost completely inhibit DNase I, while others inhibit it partially (Table 1).

Gold(III) ions (Au(III)) show inhibitory effect toward DNase I at neutral pH. Activity of DNase I is inhibited by 10 molar equivalents of Au(III) ions followed by conformational change of the enzyme molecule. Au(III) ions are coordinated to DNase I molecules. Although Au(III) ions are strong oxidants, DNase I is not oxidized or aggregated, but complexed with Au(III) ions to lose its activity. It is

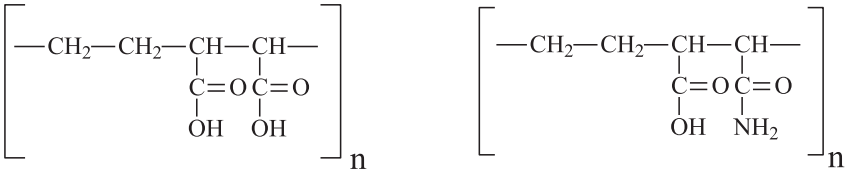


Fig. 6. Chemical structures of HEMA and AEMA.

suggested that the most likely binding sites for Au(III) ions are histidine and methionine residues in DNase I molecule. Inhibition is partially reversible by addition of the Au(III)-complexing agent thiourea when DNase I recovers 60% of its intrinsic activity [66].

Activity of exogenous DNase from protoplasts of *Daucus carota* L. var. sativa can be inhibited without affecting protoplast or cell viability. Polycations, such as poly(L-ornithine) and poly(L-lysine), form a complex with the nucleic acid and thus protect DNA, but they are not suitable inhibitors because of their toxicity to the cell membrane. The chelating agent EDTA can inhibit DNase activity but it is also not suitable because of its toxicity to plant cell protoplasts. On the other hand, addition of 45 mM sodium citrate to the medium containing protoplasts of *D. carota* at low temperature (1 °C) results in effective inhibition of DNase activity for at least 4.5 h and thereby 90–95% of the carrot protoplasts remain viable [67].

Commercial pancreatic RNase preparations are used for RNA degradation when DNA isolation is performed. Those RNase preparations may contain DNase as a contaminant and thus can harm the isolation of DNA. An effective and simple method for the inhibition of DNase is dissolving the DNA and RNA pellet and also RNase in modified standard saline citrate (SSC) buffer which contains 0.1 M sodium chloride and 0.05 M sodium citrate (pH 7.6). This method is time-saving and does not require heating or cooling for the enzyme inactivation. In the modified SSC buffer, DNA remains intact at room temperature for one month and it is also protected against a challenge of 0.4 units of purified DNase [68].

6. Physico-chemical properties of DNase inhibitors

DNase inhibitors encompass extremely versatile chemical structures ranging from the natural protein inhibitors to the synthetic low molecular weight compounds and inorganic salts and ions. All these compounds differ significantly by their physico-chemical properties due to completely different mechanisms of action. In this sense, a uniform classification of their properties is not feasible, but however a summary on the physico-chemical properties of the natural and synthetic low molecular weight inhibitors might give useful insights for further chemical modification. The lipophilicity, molecular size, flexibility and presence of hydrogen-donor and acceptors are connected to the pharmacokinetic behavior of the compounds in living organisms, including their bioavailability and transportation through different membranes to the site of action, metabolism and elimination.

The physico-chemical properties of the low molecular weight DNase inhibitors, discussed in the above sections, were calculated using the Molinspiration tool [69]. The results are shown in Table 2. MiLogP data, calculated as fragment-based contributions, indicate that the lipophilicity of the reported compounds is extended in a very broad range. The lower end is represented by the hydrophilic and negatively charged NTSB with −2.37, while the most lipophilic are the larger triazine derivatives DR396, DF365, and Eosin Yellowish with miLogP of 5.57–7.13. The amino acid derivative FDCO also shows a miLogP above 5. In the preliminary screening of new drug candidates, the LogP values <5 are established as an indicator for expected sufficient oral bioavailability [70].

On the other hand, most of the reported low molecular weight DNase inhibitors are aromatic compounds with relatively rigid structure. The conformational flexibility, described by the number of rotatable bonds, is an important factor for the optimal bioavailability [71]. The number of rotatable bonds is between 0 and 8 for the compounds summarized in Table 2. None of the molecules show more than 10 rotatable bonds which are regarded as another sign for expected good oral bioavailability.

The oral bioavailability [71] and drug transport properties [72,73] are characterized also by the polar surface area. It is expressed here as topological surface area (TPSA) which is a sum of the surface areas occupied by the oxygen and nitrogen atoms and the hydrogens attached to them. TPSA represents the hydrogen bonding capacity of the molecules. Molecules with TPSA less than 140 Å^2 are recognized to have good intestinal absorption, and those with TPSA less than 60 Å^2 show good blood–brain barrier penetration [72,73]. As could be seen in Table 2, except for PV6R, all presented compounds are expected to have good intestinal absorption, but only a limited number of them – good blood–brain barrier penetration.

Table 2
Calculated molecular properties of low molecular weight DNase inhibitors.

Compd.	m_ilogP[a]	TPSA[b]	N_{atoms}[c]	MW[d]	N_{ON}[e]	N_{OHNH}[f]	$N_{rotb.}$[g]	Vol[h]
Curcumin	2.30	93	27	368	6	2	8	332
Pyridoxal	−0.10	70	12	167	4	2	2	148
Pyridoxal 5′-phosphate	−0.76	116	16	247	7	3	4	193
NTCB	2.12	106	15	224	6	1	3	169
NTSB	−2.37	143	17	277	8	0	4	186
Nitrogen mustard	1.55	3	8	156	1	0	4	136
Crystal violet	1.46	9	28	372	3	0	4	378
DR396	5.57	138	34	495	9	3	4	377
DF365	5.57	138	34	495	9	3	4	377
Eosin Yellowish	7.13	87	29	647	5	2	2	350
Fluorescein	3.92	87	25	332	5	2	2	279
s-Triazine	−0.70	38	6	81	3	0	0	71
R282049	3.61	50	15	241	4	1	2	182
2-Amino-4, 6-dichloro-*s*-triazine	1.10	64	9	164	4	2	0	109
PV6R	0.53	179	32	472	10	2	4	351
FDCO	5.61	75	29	393	5	2	7	369

[a] Octanol-water partition coefficient, calculated by the methodology developed by Molinspiration.
[b] Polar surface area.
[c] Number of nonhydrogen atoms.
[d] Molecular weight.
[e] Number of hydrogen-bond acceptors (O and N atoms).
[f] Number of hydrogen-bond donors (OH and NH groups).
[g] Number of rotatable bonds.
[h] Molecular volume.

The hydrogen bonding capacity of the inhibitors, described by the number of H-bond donors and acceptors, differ significantly – they show 1 to 10 H-bond acceptors and 0 to 3 H-bond donors. The negatively charged dye PV6R shows the highest hydrogen bonding capacity with 10 H-bond acceptors and 2 H-bond donors. The molecular volumes of the compounds are less than 400 Å^3, even for the large Eosin Yellowish having molecular weight above 600.

Yamada et al. [9] concluded that DNase inhibitors may become novel lead compounds for development of new pharmaceuticals for the treatment of sepsis, cerebral ischemia and other inflammatory diseases. Nonsteroidal anti-inflammatory drugs, which are the most frequently used drugs for the treatment of inflammation and pain, are usually given orally. Due to calculated physico-chemical properties given in Table 2, we can conclude that most of the DNase inhibitors have good oral bioavailability and good intestinal absorption which make them suitable, from the pharmacokinetic point of view, for the treatment of sepsis and other inflammatory diseases. On the other hand, calculated physico-chemical properties show that only a limited number of DNase inhibitors have good blood–brain barrier penetration (such as *s*-triazine and R282049). By their chemical modification it may be possible to obtain new compounds with better blood–brain penetration, and thus contribute to their use in the treatment of cerebral ischemia.

7. Conclusions and future perspectives

DNases are enzymes important for normal cell function and programmed cell death. Their optimal activity is controlled by

DNase inhibitors which normally exist in living cells. Thus, increased or decreased activity of either DNase or DNase inhibitors may cause changes in many metabolic processes leading to development of various diseases. A large number of various compounds may act as DNase inhibitors. They differ in origin (natural – from humans, animals, plants, microorganisms; or synthetic), chemical structure, efficiency, strength and selectivity. Some of the inhibitors are specific toward only one type of DNase (e.g. ICAD is specific CAD inhibitor [47], NTCB and NTSB are specific DNase I inhibitors [55,56], while DR396 is a specific DNase γ inhibitor [62]), while others are active toward two (e.g. SNP and peroxynitrite toward DNase II and CAD [64]) or more types of DNase (e.g. a *M. echinospora* metabolite toward DNase I, DNase II and DNases from *Streptomyces* [33]). They also differ in mechanism of action: some of them form stable complexes with DNases (e.g. actin [1]), block the active site of the enzyme (e.g. curcumin [44]), cause the enzyme covalent modification (e.g. NTSB [56]) or fragmentation (e.g. NTCB [55]), while others form complexes with DNA rather than directly affect DNase (e.g. hydroxybiphenyls [57]). Some DNase inhibitors cause reversible (e.g. NTSB [56]), while others cause irreversible (e.g. NTCB [55]) inhibition.

DNase inhibitors differ significantly in their physico-chemical properties leading to their different pharmacokinetical behavior in living organisms. Due to calculated physico-chemical properties, we can conclude that most of the DNase inhibitors have good oral bioavailability, good intestinal absorption, but only a limited number have good blood–brain barrier penetration. As Yamada et al. [9] suggested the use of DNase inhibitors in the treatment of sepsis, cerebral ischemia and other inflammatory diseases, it can be concluded that most of them are suitable, from the pharmacokinetic point of view, for the treatment of sepsis and other inflammatory diseases. Physico-chemical properties of DNase inhibitors might give useful insights for further chemical modification and obtaining of new compounds with better blood–brain penetration, suitable in the treatment of cerebral ischemia.

Most of the inhibitors described in this review are active toward DNase I and are of natural origin. Since the number of known DNase inhibitors from plants is quite low (e.g. a protein from *N. tabacum* [34]), more attention should be given to the discovery of new DNase inhibitors from plant sources. In some papers the relationship between chemical structure and activity of synthetic DNase inhibitors was discussed [57,59,63]. Due to rapid development of organic synthetic chemistry and drug design it can be expected that the number of specific DNase inhibitors will increase in the coming years. Further, CAD is the major endonuclease involved in apoptosis, controlled and complexed with normally occurring inhibitor ICAD, but the number of known synthetic CAD inhibitors is quite low. In our opinion, due to the importance of CAD's role in cell function, further investigations should be based on the synthesis and design of new compounds able to control CAD activity.

Acknowledgments

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