Azerbaijan Pharmaceutical and Pharmacotherapy Journal
Received 12 November 2023, Accept 14 January 2024 Publish 29 February 2024

DOI https://doi.org/10.61336/appj/23-1-3

Determination of Non-Cytotoxic Concentrations of Purine Analogues on Different Types of In Vitro-Incubated Embryonic Cells: A Pilot Study

Iskra Sainova[1,*], Vera Kolyovska[1], Dimitrina Dimitrova-Dikanarova[2] and Tzvetanka Markova[3]
[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Bulgaria.
[2]Department of Biology, Medical University of Sofia, Bulgaria.
[3]Department of Pharmacology and Toxicology, Medical University of Sofia, Bulgaria.

Corresponding author: Iskra Sainova (e-mail: iskrasainova@gmail.com).

Abstract: Different concentrations of the methylxanthine/purine analogues aminophylline and 61-tartrat, were tested on in vitro-incubated embryonic avian cells from duck line DEC 99, as well as of mammalian embryonic cells from bovine line EBTr and mouse fibroblast line 3T3. In all cases, the CC_{50}/ml cell suspension, presenting the cytotoxic concentration, in which were observed 50% death or changes, was determined. On the avian embryonic cells, the determined values of the methylxantine/purine analogues were 2.4x 10^{-6} M/L about the aminophylline and 2.1x 10^{-6} M/L about the 61-tartrat. The assessed CC_{50}/mL of the same compounds on the mammalian cells were 2.4x 10^{-5} M/L about the aminophylline and 2.1x 10^{-5} M/L about the 61-tartrat, respectively. The embryonic mammalian cells were more resistant to both substances than the embryonic avian cells. On the other hand, the assessed values of both CC_{50}/ml and maximal non-toxic concentration (MNC - in which no cellular mortality or other changes can be detected) of each one of the two methylxantine/purine analogues were very near about EBTr and 3T3 mammalian cell lines, and the mouse cells, which are proved as widely used experimental in vitro-model, showed some advantages in both cases. In this way, the current data suggest a possibility about application of methylxantine/purine derivatives about genetic and/or epigenetic reparations, on DNA- and mRNA-levels of low differentiated mammalian cells.

Key Words: Mammalian embryonic cells, Avian embryonic cells, Methylxantine/purine derivatives, Possibilities about genetic and epigenetic reparations

I. INTRODUCTION

In the last years, an important role of DNA base modifications has been proposed in the regulation of genome processes by directly modulating DNA double helix stability to impede (cytosine methylation) or facilitate (methylation oxidation) access and unwinding of double-stranded DNA [1]–[3]. Other recent data have revealed the role of nucleotide and nucleoside modifications on the functions of RNA-transcripts (mRNAs), as well as on some transport RNAs, influencing in this way physiological and pathological processes [4]. Recently, p16 gene promoter methylation has been determined as a diagnostic marker in cancer of lung cancer [5]. The influence of synthetic nucleotide analogs on the stability of polymorphic G-quadruplexes has been proved as usable in engineering a required stable G-quadriplex topology, thus providing indications about different cellular events [6].

Besides their role as monomeric precursors of nucleic acids DNA and RNA, purines have been found to perform many other important functions in the cell, as modulation of energy metabolism and signal transduction, structural components of some co-enzymes and in the physiology of platelets, muscles, neurotransmission, but also about the processes of growth, proliferation and survival of all types of cells [7]. In normal physiological conditions the enzymes involved in the purine metabolism maintain in the cell a balanced ratio between their synthesis and degradation. Uric acid has been proved as the final compound of purines catabolism only in humans, unlike in the all other mammals, possessing the enzyme uricase, which has been found to convert the uric acid to easily eliminated through urine allantoin. The over-production of uric acid has been established to lead to some human diseases as podagra, as well as kidney and cardiovascular disorders, including vascular inflammation and atherosclerosis, and as a main diagnostic marker has been

determined the increased serum levels. All other mammals possess the enzyme uricase that converts uric acid to allantoin that is easily eliminated through urine.

As the most relevant pathway, promissing significant perspectives for a better pharmacological approach in the treatment of hyperuricemia-related vascular and non-vascular pathologies, has been suggested the enzyme xanthine oxidoreductase (XOR), catalyzing the two terminal reactions of purine catabolism in humans. In-depth studies on the metabolism of nucleotides in the last years have revealed their participation in many metabolic processes, influencing in this way many key functions, including enhanced immune response [8]. In this way, targeting nucleotide metabolism gives a possibility about indirect anti-microbial and anti-malignant reaction by indirect pathways as for instance (1) immune response update by maintaining the concentrations of necessary metabolites as adenosine and/or ATP, (2) increased mutability and genomic instability by disrupting the purine and pyrimidine pool, and (3) by influencing of various regulation mechanisms By application of targeting nucleotide metabolism combined with immunotherapy have achieved successful preclinical results have been achieved. A cellular signaling pathway, co-responding to various extra-cellular DNA-derived metabolites, coupling nucleoside catabolism to cellular IFN-β production by adenosine deaminases has recently been proved [9].

De novo-synthesis of nucleotides generates many important substances as for instance nucleoside monophosphates (AMP, UMP, etc.), further processing to all purine and pyrimidine nucleotides involved in multiple cellular processes, including the synthesis of nucleic acids (DNAs and RNAs). In opposite, catabolism of these compounds results in formation of nucleosides, which are further degraded by nucleoside hydrolase to nucleotide bases. Nucleosides and nucleotide bases can be exchanged between cells and tissues by various transport proteins. Among the best biological systems about both intense nucleoside metabolism and metabolism-independent uptake to terminate neuromodulator effects of nucleosides (as adenosine and guanosine) have been determined the astrocytes [10].

Astrocytes synthesize the nucleotides AMP, ADP and ATP from the nucleoside adenosine, as well as the nucleotide GTP from the nucleoside guanosine, respectively, but on the other hand, adenosine and guanosine perform functions as neuromodulators. In this relation, by tritiated thymidine, formycin B, guanosine and adenosine has been demonstrated a fast diffusional uptake of all four nucleosides, as well as a slight, Na+-independent and probably metabolism-driven uptake of thymidine (consistent with DNA-synthesis), active metabolism-driven uptake of guanosine (consistent with synthesis of DNA, RNA, but also GTP) and of adenosine (consistent with rapid nucleotide synthesis) and Na+-dependent uptake of adenosine (consistent with its concentrative uptake) and guanosine, rendering neuromodulator uptake independent of nucleoside metabolism. In in vitro-incubated primary cultures of astrocytes and neurons, guanine and guanosine have been taken up into both types of cells by the equilibrative nucleoside transporter 2 (ENT2), and their extra-cellular concentrations have been regulated mainly by astrocytes to maintain brain physiology [11].

Astrocytes as cells supplemented with various receptors for neurotransmitters and neurohormones, which allows in respective appropriate activation triggering of intra-cellular signals mediated besides by ions as by Ca2+, Na+, also by some nucleotides as cyclic AMP (cAMP). Different proteins, which are involved in the control of nucleotides and nucleosides homeostasis in the brain, suggest possibilities about the development of new therapeutic strategies against neurodegenerative diseases and the associated with them [12]. The movement of nucleosides and nucleic bases across the cellular membranes is facilitated by the nucleoside transporter proteins (NTPs), which function and activity could be regulated by various factors [13]. NTP-mediated transport has been determined as vital for the synthesis of nucleic acids in cells with lacking de novo-purine synthesis. These proteins have also been proposed to play physiological role in the transcriptomic response triggered by nucleoside analogs in malignant cells [14]. Purinergic signaling has also been characterized as an important regulatory mechanism in many different diseases and biological functions, with important implications for blood and vascular disorders [15].

Despite nucleotides and nucleosides are famous for their intra-cellular role as building "blocks" for the genetic code and/or cellular energy currencies, in the extra-cellular space they have primarily been established as signaling molecules by activation of purinergic receptors. A key role of activated by adenosine and ATP receptors in the myocardium cells has been suggested in chronic heart failure, ischemia and reperfusion, but on the other hand, in cardiac protection against myocardial infarction and arrhythmias [16]. Additionally, many adenoside and ATP receptor have been found to regulate the fate of stem/progenitor cells to proliferation, or to senescence and apoptosis, respectively, by decreasing p53 and Rb through cAMP-PKA/Rac1/p38 MAPK pathway [17].

The role of purinergic signals about the liver homeostasis, restriction of inflammation, stimulation of liver regeneration, modulation of fibrogenesis, has also been discussed, and thus, development of targeted therapeutic strategies against liver diseases based on purinergic signals by blocking of nucleotide receptors, by enhancement of ectonucleoside triphosphate diphosphohydrolase activity and/or by activation of adenosine receptors. Guanosine triphosphatases (GTPases), belonging to the Rho-family, have been proved to regulate cellular signaling and cytoskeletal dynamics, thus playing a pivotal role in the processes of cellular adhesion, migration and cell cycle progression, and pathogenic variations, affecting these biological processes, have been implicated in various neurodevelopmental pathologies. Additionally, the modulation of purinergic signaling has been proposed as a novel approach to preventing or diminishing of fibrosis in injuries of internal tissues and organs, including

in the maintenance of the functions of the intestinal mucosa, bone marrow hematopoietic stem cells (HSCs) and immune system [18].

The influence of many nucleotide analogs on the secretion of vascular endothelial growth factor (VEGF) by keratinocytes and fibroblasts, but also their influence on the viability and proliferation of keratinocytes, fibroblasts and endothelial cells have also been analyzed [19]. Nucleotide metabolism supports the processes of DNA-replication and RNA-synthesis, enabling in this way cell growth and division/proliferation, which have been inhibited in nucleotide depletion and disbalance [20]. According to the same authors, imbalanced nucleotide levels are not detected until S phase, rendering cells reliant on replication stress signaling to cope with this metabolic problem, which leads to disrupted coordination of cellular growth and division. As particularly important about the balance of phosphates in the nucleotides has been proved Mg^{2+} - 1.1 mM of the free Mg^{2+} and 8.0 mM of the bound in complexes with Mg, respectively [21].

The molecular design, synthesis and functional evaluation are described triplex-forming oligonucleotides, 2-amino-2'-deoxy-nebularine derivatives (novel artificial nucleoside analogues), have been similarly described and proceeded [22]. Duplex DNA, bearing the 5-methyl-2'-deoxycytosine and 2'-deoxyguanosine base pair has recently been recognized by triplex DNA formation. On this basis, triplex-forming oligonucleotides have suggested a possibility about molecular design, synthesis and functional evaluation. Differences in this balance in the different cellular compartments have been assessed for instance, these values have been found to be between the cytoplasm and mitochondria, but not between the cytoplasm and the nucleus. A general strategy about the incorporation of modified nucleosides into the cellular RNA, expanding the chemical toolkit of modified bases for studying dynamic RNA-behavior in the living cells, has recently been proposed [23].

The concentration changes of nucleosides and nucleotides in biological samples have shown possibility about application about discovery of pathological events and/or about elucidation of disease mechanisms [24], including in the different types of malignancies [25]. For this goal the authors have used phase chromatography-mass spectrometry (LC-MS) method for simultaneous quantification. On this basis, opportunities nucleoside analogues to target specific gene expression have been proposed [26]. Recent works have been directed to the application of click chemistry methodology in the field of nucleosides, nucleotides and nucleic acids about pharmacological applications [27]. Tissue-specific and condition-specific expression patterns have been suggested among the promising tools for nucleic acid-based therapeutic applications to increase potency, duration and safety have been proposed the 2'-F/Me nucleotides [28].

As the most prevalent, abundant and conserved internal co-transcriptional modification in eukaryotic RNAs, especially within higher eukaryotic cells, has again been characterized N^6-methyladenosine (m^6A), in both normal functions and pathologies [29] in the regulation of immune system and autoimmune diseases [30], but also in many disabilities, malignancies [31], [32], including leukemias [33], congenital dysplasia [34] and in aging regulation [35], but on the other hand, this modification has been determined as biomarkers and therapeutic targets. The role of m^6A modification and coded by it proteins, as well as regulators of the pathways, in which they participate, in autophagy-related mechanisms has been shown [36]. As one of the main mechanisms of action has been established to be by selection of crucial to alternative splicing degenerate 5'-splice sites (by small non-coding RNA m^6A (snRNA m^6A) [37]. Aberrant m^6A methylation has been proved in many in cardiovascular diseases (CVDs), including cardiac hypertrophy, heart failure, arterial aneurysm, vascular calcification and pulmonary hypertension [38]. The restoration of the m^6A modification balance via targeting specific imbalanced regulators has been proposed as a new anti-malignant strategy [39].

II. MATERIALS AND METHODS

A. CELL CULRUTES

Avian and mammalian embryonic cells from lines DEC 99, EBTr and 3T3, derived from duck embryo, embryonic bovine trachea and mouse embryonic fibroblasts, respectively, were incubated in a humidified 5% CO_2/95% air incubator at 37∘C. Cells of all types (in initial volume 3x10^4 on 1 ml cultural fluid) were routinely grown in a growth medium, a combination of Parker-E199 (Sigma) and Iskov's modification of Dulbecco's medium (IMDM - Sigma), in ratio 1:1, supplemented with 25 mM HEPES buffer (Sigma), 5% normal bovine serum (Sigma), and antibiotics in volumes (100 IU/ml Penicillin - Sigma and 100 μg/ml Streptomycin - Sigma) were added.

B. DETERMINATION OF THE VALUES OF MND AND CC_{50} OF IN VITRO-INCUBATED EMBRYONIC CELLS

Semi-confluent monolayers of all cellular types were treated with different dilutions of the methylxantine/purine derivatives aminophylline (Sigma) and 61-tartrat (Sigma), and the values of both maximal non-toxic concentration (MNC) (the highest concentration of the respective tested substance, in which no cellular mortality or other changes can be detected) and CC_{50}/ml cell suspension (in which 50% of the treated in vitro-incubated cells were dead or changed were determined. For this goal, gradual dilutions of each one of the substances were previously prepared. All cells were observed under invert microscope Televal at 24-hour intervals. The cellular viability cells was assessed by Trypan Blue Dye Exclusion Test, after trypsinization and resuspendation. The test is based on the capability of the intact membranes of the viable cells to exclude the dye, unlike the unviable (dead) cells [40]:

$$\%\text{cell viability} = \frac{\text{total number of viable cells per 1 ml cell suspension}}{\text{total number of cells per 1 ml cell suspension}} \times 100.$$

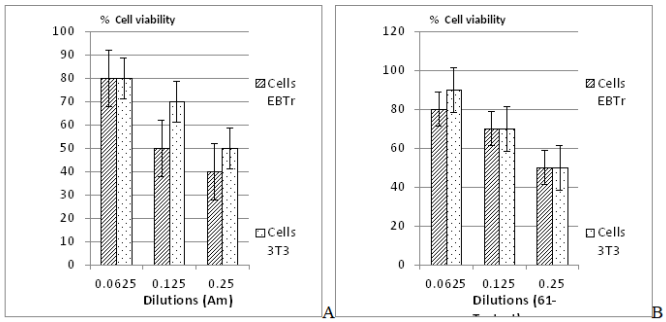

FIGURE 1: CD_{50}/ml of the methylxantine/purine derivatives aminophylline (A) and 61-Tartrat (B) on the mammalian embryonic cells from lines EBTr and 3T3 and % of cell viability in different concentrations of each one of the two compounds on each one of the two mammalian cellular types

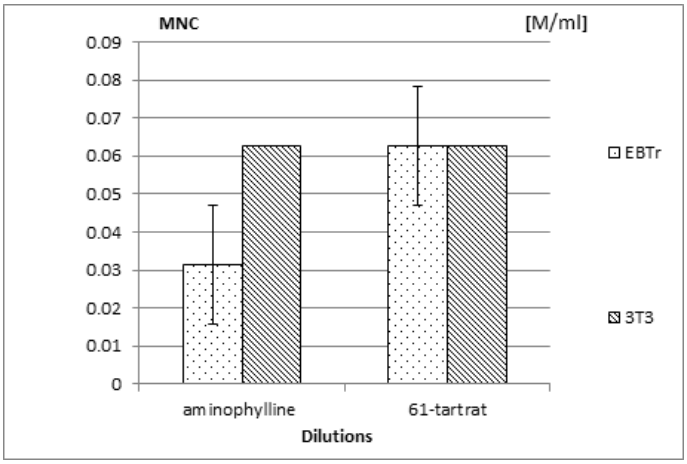

FIGURE 2: Maximal non-toxic concentrations (MNC) of the methylxantine/purine derivatives aminophylline and 61-Tartrat on the embryonic mammalian cell lines EBTr and 3T3

III. RESULTS AND DISCUSSIONS

The embryonic mammalian/bovine cells were more resistant to the two tested compounds than the embryonic avian/duck cells (Table 1).

Despite of the near values of the CC_{50}/ml (the concentration, in which can be detected 50% mortality or other changes in the treated cells) and the MNC (Figure 1) and of the CC_{50}/ml (Figure 2) of each one of the two methylxantine/purine analogues were near about both EBTr and 3T3 mammalian cell lines, the mouse cells showed some advantages in both cases.

Identical elements in embryonic cells from the same three species have been established in the so called conserved regions (CRs) of homology, and as a prove about this has been determined the noted high number of CCC(A/T)CCC motifs, which are localized in the Oct-4 upstream promoter sequences of the cellular genomes [41]. On the other hand, high degree of homology in many germline genes between cattle and mouse has been revealed [42]. Messages about conserved transcriptomic characteristics between human and cattle [43], as well as between human and mouse [44] have recently been received.

As a usable strategy about reduction the toxicity of nucleoside and nucleotide analogues in their applications as drugs, has recently been proposed their delivery into nanoparticles [45]. The separate fragments (small azide compounds) have been found to cause small perturbations to the geometry of the azide moiety, but they apparently alter atomic charge distributions and molecular electrostatic potentials, unlike the whole molecule of AZT [46]. Taking all these features in consideration, in the last years, nucleic-acid-based small molecule and oligonucleotide therapies have been determined as attractive topics due to their potential for effective target of various disease-related modules and specific control of specific gene expression in respective disease, but also in a concrete organism [47]. Although all the chiral centers in the backbone have been characterized as mirror converted of the natural D-nucleic acids, the L-nucleic acids have been found to be equipped with the same nucleobases (A, G, C and U in RNA or T in DNA, respectively), which have been characterized as critical to be maintained the programmability and to form adaptable tertiary structures for target binding in the processes of replication and transcription.

By taking in consideration the functions of particularly nucleotides as metabolites and regulatory molecules in the epigenetic regulation and biological processes, a key role of metabolism in epigenetics as a critical regulator of biological events has been underlined [48]. Different mechanisms, by which DNA-modifications and damage might perturb the epigenetic patterns have been proved [49].

The replacement of C with base analogs, leading in this way to appearance of inhibitory complexes with methyltransferases has been found to alter modestly the methyl-binding domain (MBD) affinity and thus, perturbing the MBD-DNA binding, which has proposed that these analogues probably perturb the epigenetic patterns mainly by direct inhibition of the methyltransferases. In opposite, base analogues with an increased tendency to form base mis-pairs (as BrU), have been suggested to cause epigenetic changes by enhanced MBD-DNA binding, but not through direct influences on the methyltransferases. Unlike the large DNA- and RNA-molecules, these substances have shown comparatively easy transport through the blood-testicular barrier (BTB) [50], blood-intestinal barrier (BIB) [51] and blood-brain barrier (BBB) [52]. Dietary nucleotides supplementation has also been found to support the antioxidant status and by prevention of the intra-uterine growth retardation (IUGR) on the oxidative status and mitochondria DNA damage through improving both non-enzymatic and enzymatic antioxidant capacities as well as mitochondria biogenesis [53].

The different chemical modifications, which have been identified in the cellular nucleic acids and in particular in the various types of cellular RNAs have been presented as a new level in the control of genetic information [54]. Such modifications in the mRNAs could affect protein production

CC_{50}/ml	Dilutions Aminophylline (mg/ml)	Dilutions 61-Tartrat (mg/ml)
avian cells from duck embryo cell line DEC 99	2.4 x 10-6	2.1 x 10-6
mammalian cells from embryonic bovine trachea cell line EBTr	2.4 x 10-5	2.1 x 10-5

TABLE 1: Cytotoxicity and CC_{50}/ml of the tested methylxanthine/purine derivatives on in vitro-incubated cultures of avian cells from duck embryo and mammalian cells from bovine embryo

by influencing the splicing, and/or translation, and decay rates through various mechanisms. The tRNAs and rRNAs have been found to require often modification for proper biogenesis and stability, but also to utilize base alterations and therefore, tune structure and function. Modifications in all RNA species have been linked to various diseases. As potential biomarkers and therapeutic targets in this relation have been proved the lncRNAs [55].

m^6A modification and its protein products have also been suggested as markers and targets for diagnosis and treatment of female reproductive dysfunction [56]. Correlation between m^6A modification and the tumor immune landscape in cases with clear cell renal cell carcinoma (ccRCC) has been proved [57], but also in colorectal cancer and associated with the last inflammatory bowel diseases [58]. In a case with breast cancer has been suggested development of program about a personalized medicine, based on the immune cell infiltration characteristics of the tumor microenvironment and the m^6A methylation modification pattern [59]. A comprehensive picture of epitranscriptomic regulatory mechanism in mouse retina has been provided on the basis of the genome-wide RNA m^6A modification profile [60]. Regulation of m^6A deposition in the molecule of mRNA, but also the influence of this modificatiom on the process of mRNA translation and/or its decay, as well as its role on non-coding chromosome-associated RNAs has been proposed as a novel mechanism of transcription regulation and in this way, possibilities about understanding and development of new methods about regulation and prevention of in disease development. Insight about eventual applications of nucleobase-modified nucleosides in the field of synthetic biology have also recently been made [61].

In experiments with young and aged mouse brains, as well as in patients with Alzheimer's disease has been observed decreased m^6A RNA methylation of synaptic genes in brain aging and in cases of dementia [62]. Taking these findings in consideration, reduced m^6A-modified transcripts have been proposed to be related with impaired synaptic protein synthesis. In this way, targeting the m^6A RNA methylation machinery has been determined as a promising strategy about prevention of cognitive decline. On the other hand, mediating mechanisms of miRNAs in m^6A modification and its regulatory proteins during the occurrence and development of various diseases has recently been discussed [63]. Despite m^6A modification has been found to be affected by transcriptional dynamics, recently has been suggested that m^6A machinery influences transcription and determines chromatin signature [64]. Assays on the interaction of m^6A and other nucleoside analogues (as 5-methylcytosine, 5-hydroxymethycytosine) have revealed possibilities about understanding, prediction and modulation of the interactions between modified nucleic acids and proteins, including at the atomic level [65].

The epigenetically-modified nucleic acids, have been proposed as a basis about discussion of the mechanisms of recognition by different proteins [66]. In this way, the binding of these compounds has also been found to induce important structural switches. In this way, the obtained RNA products containing dthG, as well as dthG together with 5-bromocytosine could function as effectively as natural sgRNAs in an in vitro CRISPR-Cas9 cleavage assay. Substitutions of G residues by isomorphic fluorescent thienoguanosine (thG) analogs, but also by 5-bromocytosine, 7-deazaadenine and 5-chlorouracil, as well as of the U residues in the RNA-molecules by ethylpseudouridine, respectively, have been shown to direct Cas9 nuclease cleavage when it is incorporated in sgRNA [67]. Thus, a possibility to be expanded the impact and therapeutic value of CRISPR-Cas9 system and other RNA-based technologies have been proposed. The transcriptional efficiency of emissive fully modified RNA was found to benefit from the use of various T7 RNA polymerase variants. Moreover, dthG could be incorporated into PCR products by Taq DNA polymerase together with the other three base-modified nucleotides.

IV. CONCLUSION

The observed one logarithm higher values of the two tested methylxantine/purine derivatives on the in vitro-incubated cultures of mammalian bovine cells than on these of avian/duck embryonic cells suggested higher resistance of the mammalian cells on the treatment with these substances. On the other hand, very near values of both CC_{50}/ml and MNC were noted between the mammalian cells with bovine origin and mouse embryonic fibroblasts from 3T3 cell line. These results suggested a possibility about application of methylxantine/purine derivatives about genetic and/or epigenetic reparations, on the DNA- and mRNA-levels of these compounds with low differentiated mammalian cells, in particular with a convenient and widely used experimental in vitro-model as mouse embryonic cells.

REFERENCES

[1] Rausch, C., Zhang, P., Casa-Delucchi, C. S., Daiβ, J. L., Engel, C., Coster, G., Hastert, F. D., Weber, P., & Cordoso, M. C. (2021). Cytosine base modifications regulate DNA duplex stability and metabolism. Nucleic Acids Research, 49(22), 12870-12894.

[2] Zhang, Y., & Kleiner, R. E. (2019). A metabolic engineering approach to incorporate modified pyrimidine nucleosides into cellular RNA. Journal of American Chemical Society, 141(8), 3347-3351.

[3] Zheng, Y. Y., Wu, Y., Begley, T. J., & Sheng, J. (2021). Sulfur modification in natural RNA and therapeutic oligonucleotides. RSC Chemical Biology, 2(4), 990-2003.

[4] Bao, Z., Li, T., & Liu, J. (2023). Determining RNA natural modifications and nucleoside analog-labeled sites by chemical/enzyme-induced base mutation principle. Molecules, 28(4), 1517.

[5] Li, Y., Zhang, T., Zhang, H., Wang, X., Liu, X., Huang, Q., & Li, L. (2020). Clinical significance of p16 gene methylation in lung cancer. Advances in Experimental Medicine and Biology, 1255, 133-142.

[6] Sagi, J. (2017). In what ways do synthetic nucleotides and natural base lesions alter the structural stability of G-quadroplex nucleic acids? Journal of Nucleic Acids, 2017, 1641845.

[7] Maiuolo, J., Oppedisano, F., Gratteri, S., Muscoli, C., & Mollace, V. Regulation of uric acid metabolism and expression. International Journal of Cardiology, 2016, 213, 8-14.

[8] Wu, H.-L., Gong, Y., Ji, P., Xie, Y.-f., Jiang, Y.-Z., & Liu, G.-y. (2022). Targeting nucleotide metabolism: a promising approach to enhance cancer immunotherapy. Journal of Hematology & Oncology, 15(1), 45.

[9] Dhanwani, R., Takahashi, M., Mathews, I. T., Lenzi, C., Romanov, A., Watrous, J. D., Pieters, B., Hedrick, C. C., Benedict, C. A., Linden, J., Nilsson, R., Jian, M., & Sharma, S. (2020). Cellular sensing of extracellular purine nucleosides triggers an innate IFN-β response. Science Advances, 6(30), 1197-1218.

[10] Peng, L., Huang, R., Yu, A.C.H., Fung, K. Y., Rathbone, M. P., & Hertz, L. (2005). Nucleoside transported expression and function in cultured mouse astrocytes. Glia, 52(1), 25-35.

[11] Nagasawa, K., Kawasaki, F., Tanaka, A., Nagai, K., & Fujimoto, S. (2007). Characterization of guanine and guanosine transport in primary cultured rat cortical astrocytes and neurons. Glia, 55(14), 1397-1404.

[12] Chang, C.-P., Wu, K.-C., Lin, C.-Y., & Chern, Y. (2021). Emerging roles of dysregulated adenosine homeostasis in brain disorders with a specific focus on neurodegenerative diseases. Journal of Biomedical Science, 28(1), 70.

[13] Podgorska, M., Kocbuch, C., & Pawelczyk, T. (2005). Recent advances in studies on biochemical and structural properties of equilibrative and concentrative nucleoside transporters. Acta Biochimica Polonica, 52(4), 749-758.

[14] Molina-Acras, M., Trigueros-Motos, L., Casado, F. J., & Pastor-Anglada, M. (2008) Physiological and pharmacological roles of nucleoside transporter proteins. Nucleosides Nucleotides Nucleic Acids, 27(6), 769-778.

[15] Idzko, M., Ferrari, D., Riegel, A.-K., & Eltzschig, H. K. (2014). Extracellular nucleotide and nucleoside signaling in vascular and blood disease. Blood, 124, 1029-1037.

[16] Wang, P., Jia, J., & Zhang, D. (2020). Purinergic signaling in liver diseases: pathological functions and therapeutic opportunities. JHEP Reports, 2(6), 100165.

[17] Scala, M., Nishikawa, M., Nagata, K.-I, & Striano, P. (2021). Pathophysiological mechanisms in neurodevelopmental disorders caused by Rac GTPases dysregulation: what's behind neuro-RACopathies. Cells, 10(12), 3395.

[18] Yamamoto, S., Wang, M. F., Adjej, A. A., & Ameho, C. K. (1997). Role of nucleosides and nucleotides in the immune system, gut reparation after injury, and brain function. Nutrition, 13(4), 372-374.

[19] Weglowska, E., Koziolkiewicz, M., Kaminska, D., Grobelski, B., Pawelczak, D., Kolodziejczyk, M., Belecki, S., & Gandaszewska-Darmach, E. (2021). Extracellular nucleotides affect the proangiogenic behavour of fibroblasts, keratinocytes, and endothelial cells. International Journal of Molecular Sciences, 23(1), 238.

[20] Diehl, F. F., Miettinen, T. P., Elbashir, R., Nabel, C. S., Darnell, A. M., Do, B. T., Manalis, S. R., Lewis, C. A., & Henden, M. G. V. (2022). Nucleotide imbalance decouples cell growth from cell proliferation. Nature Cell Biology, 24(8), 1252-1264.

[21] Traut, T. W. (1994). Physiological concentrations of purines and pyrimidines. Molecular and Cellular Biochemistry, 140(1), 1-22.

[22] Notomi, R., Wang, L., Sasaki, S., & Taniguchi, Y. (2021). Design and synthesis of purine nucleoside analogues for the formation of stable antiparallel-type triplex DNA with duplex DNA bearing the 5mCG base pair. RSC Advances, 11, 21390-21396.

[23] Zhang, Y., & Kleiner, R. E. (2019). A metabolic engineering approach to incorporate modified pyrimidine nucleosides into cellular RNA. Journal of the American Chemistry Society, 141(8), 3347-3351.

[24] He L, Wei X, Ma X, Yin, X., Song, M., Donninger, H., Yaddanapudi, K., McClain, C. J., & Zhang, X. (2019). Simultaneous quantification of nucleosides and nucleotides from biological samples. Journal of the American Society for Mass Spectrometry, 30(6), 987-1000.

[25] Ma, J., Zhong, M., Xiong, Y., Gao, Z., Wu, Z., Liu, Y., & Hong, X. (2021). Emerging roles of nucleotide metabolism in cancer development: progress and prospect. Aging (Albany NY), 13(9), 13349-13358.

[26] Ewald, B., Sampath, D., & Plunkett, W. (2008). Nucleoside analogs: molecular mechanisms signaling cell death. Oncogene, 27(50), 6522-6537.

[27] Perrone, D., Marchesi, E., Preti, L., & Navacchia, M. L. (2021). Modified nucleosides, nucleotides and nucleic acids via click azide-alkyne cycloaddition for pharmacological applications. Molecules, 26(11), 3100.

[28] Guenther, D. C., Mori, S., Matsuda, S., Gilbert, J. A., Willoughby, J. L. S., Hyde, S., Bisbe, A., Jiang, Y., Agarwal, S., Madaoui, M., Janas, M. M., Charisse, K., Maier, M. A., Egil, M., & Manoharan, M. (2022). Role of "magic" methyl: 2'-deoxy-2'-α-F-2'-β-C-methyl pyrimidine nucleotides modulate RNA interference activity through synergy with 5'-phosphate mimics and mitigation of off-target effects. Journal of the American Chemistry Society, 144(32), 14517-14534.

[29] Jiang, X., Liu, B., Nie, Z., Duan, L., Xiong, Q., Jin, Z., Yang, C., & Chen, Y. (2021). The role of m6A modification in the biological functions and diseases. Signal Transduction and Targeted Therapy, 6(1), 74.

[30] Wang, Y., Li, L., Li, J., Zhao, B., Huang, G., Li, X., Xie, Z., & Zhou, Z. (2021). The emerging role of M6A modification in regulating the immune system and autoimmune diseases. Frontiers in Cell and Developmental Biology, 9, 755691.

[31] Gu, C., Shi, X., Dai, C., Shen, F., Rocco, G., Chen, J., Huang, Z., Chen, C., He, C., Huang, T., & Chen, C. (2020). RNA m6A modification in cancers: molecular mechanisms and potential clinical applications. The Innovation, 1(3), 100066.

[32] Ma, W., & Wu, T. (2022). RNA m6A modification in liver biology and its implication in hepatic diseases and carcinogenesis. American Journal of Physiology Cell Physiology, 323(4), C1190-C1205.

[33] Rong, Y., Yashu, L., Wen, T., Zhou, F., Zhang, H. (2022). RNA m6A modification: mapping methods, roles, and mechanisms in acute myeloid leukemia. Blood Science, 4(3), 116-124.

[34] Lei, K., Lin, S., & Yuan, Q. (2023). N6-methyladenosine (m6A) modification of ribosomal RNAs (rRNAs): critical roles in mRNA translation and diseases. Genes & Diseases, 10(1), 126-134.

[35] Wu, Z., Wang, S., Belmonte, J. C. I., Zhang, W., Qu, J., & Liu, G.-H. (2022). Emerging role of RNA m6A modification in aging regulation. Current Medicine, 1, 8.

[36] Paravasivam, A., Priyadharsini, J. V. (2022). The emerging role of m6A modification in autophagy regulation and its implications in human disease. Epigenomics, 14(10), 565-568.

[37] Parker, M. T., Soanes, B. K., Kusakina, J., Larrieu, A., Knop, K., Joy, N., Breidenbach, F., Sherwood, A. V., Barton, G. J., Fica, S. M., Davies, B. H., & Simpson, G. G. (2022). m6A modification of U6 snRNA modulates usage of two major classes of pre-mRNA 5' splice site. Chromosomes and Gene Expression, Genes and Genomics, 11, e78808.

[38] Qin, Y., Li, L., Luo, E., Hou, J., Yan, G., Wang, D., Qiao, Y., & Tang, C. Role of m6A methylation in cardiovascular disease (Review). International Journal of Molecular Medicine, 2020; 46(6), 1958-1972.

[39] Gao, R., Ye, M., Liu, B., Wei, M., and Dong, K. (2021). m6A modification: a double-edged sword in tumor development. Frontiers in Oncology, 11, 679367.

[40] Strober, G. (2001). Trypan blue exclusion test of cell viability. Current Protocols in Immunology, 3B, doi: 10.1002/0471142735.ima03bs21; PMID: 18432654.

[41] Soto, D. A., Ross, P. J. (2021). Similarities between bovine and human germline development revealed by single-cell RNA sequencing. Reproduction, 161(3), 239-253.

[42] Li, G., Penagaricano, F., Weigel, K. A., Zhang, Y., Rosa, G., & Khatib, H. (2012). Comparative genomics between fly, mouse, and cattle identifies genes associated with sire conception rate. Journal of Dairy Science, 95(10), 6122-6129.

[43] Yao, Y., Liu, S., Xia, C., Gao, Y., Pan, Z., Canela-Xandri, O., Khamseh, A., Rawlik, K., Wang, S., Li, B., Zhang, Y., Pairo-Castineira, E., D'Mellow, K., Li, X., Yan, Z., Li, C.-J., Yu, Y., Zhang, S., Ma, L., Cole, J. B., Ross, P. J., Zhou, H., Haley, C., Liu, G. E., Fang, L., & Tenesa, A. (2022). Comparative transcriptome in large-scale human and cattle populations. Genome Biology, 23(1), 176.

[44] Breschi, A., Gingeras, T. R., Guigo, R. (2017). Comparative transcriptomics in human and mouse. Nature Reviews Genetics, 18(7), 425-440.

[45] Baroud, M., Lepeltier, E., Thepot, S., El-Makhour, Y., & Duval, O. (2021). The evolution of nucleosidic analogues: self-assembly of prodrugs into nanoparticles for cancer drug delivery. Nanoscale Advances, 3, 2157-2179.

[46] Chen, F.-F., Wang, F. (2009). Electronic structure of the azide group in 3'-azido-3'-deoxythymidine (AZT) compared to small azide compounds. Molecules, 14(7), 2656-2668.
[47] Dantsu, Y., Zhang, Y., & Zhang, W. (2022). Advances in therapeutic L-nucleosides and L-nucleic acids with unusual handedness. Genes, 13(1), 46.
[48] Suganuma, T., & Workman, J. L. (2021). Nucleotide metabolism behind epigenetics. Frontiers in Endocrinology (Lausanne), 12, 731648.
[49] Lao, V. V., Darwanto, A., & Sowers, L. C. (2010). Impact of base analogs on the binding of DNA by the methyl-binding domain of MeCP2 and methylation of DNMT1. Biochemistry, 49(47), 10228-10236.
[50] Kato, R., Maeda, T., Akaike, T., & Tamai, I. (2005). Nucleoside transport in the blood-testis barrier studied with primary-cultured sertoli cells. The Journal of Pharmacology and Experimental Therapeutics, 312(2), 601-608.
[51] Tsesmedzis, N., Paulin, C.B.J., Rudd, S.G., & Herold, N. (2018). Nucleobase and nucleoside analogues: resistance and re-sensitization at the level of pharmacokinetics, pharmacodynamics and metabolism. Cancers (Basel), 10(7), 240.
[52] Cansev, M. (2006). Uridine and cytidine in the brain: their transport and utilization. Brain Research Reviews, 52(2), 389-397.
[53] Hu, L., Peng, X., Qin, L., Wang, R., Fang, Z., Lin, Y., Xu, S., Feng, B., Wu, D., & Che, L. (2018). Dietary nucleotides supplementation during the suckling period improves the antioxidative ability of neonates with intrauterine growth retardation when using a pig model. RSC Advances, 8(29), 16152-16160.
[54] Rondtree, I. A., Evans, M. E., Pan, T., & He, C. (2017). Dynamic RNA modifications in gene expression regulation. Cell, 169(7), 1187-1200.
[55] Statello, L., Guo, C.-J., Chen, L.-L., & Huarte, M. (2021). Gene regulation by long non-coding RNAs and its biological functions. Nature Reviews Molecular Cell Biology, 22(2), 96-118.
[56] Chen, J., Fang, Y., Xu, Y., & Sun, H. (2022). Role of m6A modification in female infertility and reproductive system diseases. International Journal of Biological Sciences, 18(9), 3592-3604.
[57] Zhong, J., Liu, Z., Cai, C., Duan, X., Deng, T., & Zeng, G. (2020). m6A modification and tumor immune landscape in clear cell renal carcinoma. Journal for ImmunoTherapy of Cancer, 9(2), e001646.
[58] Xu, X., Huang, J., Ocansey, D. K. W., Xia, Y., Zhao, Z., Xu, Z., Yan, Y., Zhang, X., & Mao, F. (2021). The emerging clinical application of m6A RNA modification in inflammatory bowel disease and its associated colorectal cancer. Journal of Inflammation Research, 4, 3289-3306.
[59] Liu, F., Yu, X., & He, G. (2021). m6A-mediated tumor invasion and methylation modification in breast cancer microenvironment. Journal of Oncology, 2021(6), 9987376.
[60] Yan, D., Luo, G., Chen, X., & Xu, W. (2021). A genome-wide map of m6A modification in mouse retina. Investigative Ophthalmology & Visual Science, 62(8), 1572.
[61] Berdis, A. (2022). Nucleobase-modified nucleosides and nucleotides: applications in biochemistry, syntethic biology, and drug discovery. Frontiers in Chemistry, 10, 1051525.
[62] Castro-Hernandez, R., Berulava, T., Metelova, M., Epple, R., Centeno, T. P., Richter, J., Kaurani, L., Pradhan, R., Sakib, M. S., Burkhardt, S., Ninov, M., Bohnsack, K. E., Delalle, I., & Fischer, A. (2023). Conserved reduction of m6A RNA modifications during aging and neurodegeneration is linked to changes in synaptic transcripts. Proceedings of the National Academy of Sciences of the United States of America, 120(9), e2204933120.
[63] Mei, Z., Mou, Y., Zhang, N., Liu, X., He, Z., & Gu, S. (2023). Emerging mutual regulatory roles between m6A modification and microRNAs. International Journal of Molecular Sciences, 24(1), 773.
[64] Akhtar, J., Logoboni, M., & Junion, G. (2021). m6A RNA modification in transcription regulation. Transcription, 12(5), 266-276.
[65] Hajnick, M., Alonso-Gil, S., Polyansky, A. A., de Ruiter, A., & Zagrovic, B. (2022). Interaction preferences between protein side chains and key epigenetic modifications 5-methylcytosine, 5-hydroxymethylcytosine and N6-methyladenine. Scientific Reports, 12(1), 19583.
[66] Hardwick, J. S., Lane, A. N., & Brown, T. (2018). Epigenetic modifications of cytosine: biophysical properties, regulation and function in mammalian DNA. BioEssays, 40(3), 1700199.
[67] Yang, H., Eremeeva, E., Abramov, M., Jacquemyn, M., Groaz, E., Daelemans, D., & Herdewijn, P. (2023). CRISPR-Cas9 recognition of enzymatically synthesized base-modified nucleic acids. Nucleic Acids Research, 51(4), 1501-1511.