ACTA ABP BIOCHIMICA POLONICA

Vol. 63, No 4/2016
709–716
https://doi.org/10.18388/abp.2016_1363

Review

Selected small molecules as inducers of pluripotency

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The general idea of regenerative medicine is to fix or replace tissues or organs with live and patient-specific implants. Pluripotent stem cells are capable of indefinite self-renewal and differentiation into all cell types of the body. An easily accessible source of induced pluripotent stem cells (iPSCs) may allow obtaining and culturing tissues *in vitro*. Many approaches in the methods leading to obtain iPSCs have been tested in order to limit immunogenicity and tumorigenesis, and to increase efficiency. One of the approaches causing pluripotency is usage of small molecule compounds. It would be of great importance to assess their specific properties and reveal their new capacity to induce pluripotent stem cells and to improve reprogramming efficiency. Identification of the epigenetic changes during cellular reprogramming will extend our understanding of stem cell biology and many therapeutic applications. In this paper we discuss mainly the nucleotide derivatives, already proven or for now only putative inducers of the cells' pluripotency, that modulate the epigenetic status of the cell.

Key words: reprogramming, pluripotency, small molecules, iPSCs

Received: 06 June, 2016; revised: 24June, 2016; accepted: 24 August, 2016; available on-line: 26 October, 2016

INTRODUCTION

Cells produced with a renowned method requiring overexpression of four transcription factors: Oct4, Sox2, Klf4, and c-Myc (the so-called Yamanaka factors) have been named induced pluripotent stem cells (iPSCs) (Takahashi & Yamanaka, 2006; Takahashi *et al.*, 2007). They are very similar to embryonic stem cells (ESCs) with respect to morphology, phenotype, transcription and epigenetics (Takahashi & Yamanaka, 2006; Takahashi *et al.*, 2007). Both types of these cells share a similar potency, differentiability and cell division rate. Furthermore, they are able to aggregate into embryoid bodies (Takahashi & Yamanaka, 2006).

There are a number of assays verifying pluripotency of stem cells. The most popular *in vivo* teratoma assay (Gertow *et al.*, 2007; Wesselschmidt, 2011) is based on injecting potentially pluripotent stem cells into various sites of an immunocompromised mouse body followed by the growth of a tumor. When the injected cells are pluripotent, the tumor demonstrates characteristics of a teratoma, namely the development of differentiated cells originated at all three germ layers (ectoderm, mesoderm, and endoderm) (Brivanlou *et al.*, 2003). The weakness of this assay is lack of standardization, and the time, cost and labor consumption. Moreover, the greatest disadvantage refers to the usage of experimental animals (Hentze *et al.*, 2009; Wesselschmidt, 2011). One of alternative tests is analysis of expression of the pluripotency-associated markers (Fong *et al.*, 2008; Mitsui *et al.*, 2003; Pesce & Scholer, 2001) and exploitation of epigenetic modifications responsible for pluripotency. Another method is based on *in vitro* embryoid bodies' models of spontaneous and directed differentiation, e.g. cardiac bodies from isolated cardiac cells can be generated that give rise to cardiomyocytes, endothelial cells and smooth muscle cells (Höbaus *et al.*, 2013; Taubenschmid & Weitzer, 2012). To fill the gap between the *in vivo* and *in vitro* systems, the *in silico* models supported by genome wide data sets are used to help identifying characteristic features of pluripotent stem cells in functional genomics (Müller *et al.*, 2008, 2011; Williams *et al.*, 2011). An alternative *in vivo* system uses chicken eggs in which stem cells are transplanted onto chorioallantoic membrane of the chicken embryo and then a tumor similar to teratoma may arise (Durupt *et al.*, 2012; Hagedorn *et al.*, 2005). Another option to study pluripotency is an *in situ* analysis. In this organotypic model, stem cells are injected into a tissue, such as skin, and then either their development and differentiation or repopulation of cells leaving behind an extracellular matrix by stem cells are observed (Ott & Taylor, 2006; Elliott *et al.*, 2012).

Miscellaneous and numerous methods have evolved to reprogram somatic cells. A lot of improvements in these methods have been made in order to solve problems associated with a derived iPSC line, and thus to limit immunogenicity and tumorigenesis, and increase efficiency (Zhao *et al.*, 2011; Ma *et al.*, 2013). One of the concerns is that the stresses of reprogramming might lead to deleterious DNA mutations in the iPSC lines (Bhutani *et al.*, 2016). Recent studies have demonstrated that reprogramming-based mutations are generally benign and it is improbable to introduce mutational variants that would make cells inadequate for therapy (Bhutani *et al.*, 2016). The acquisition of a stable pluripotent state appears to be difficult to control (Pennarossa *et al.*, 2013). iPSCs and cancer cells share many similarities, like high proliferation rate, immortal cell growth, similarities in gene expression signature, in epigenetic status and chromosomal instability (Bernhardt *et al.*, 2012).

All methods leading to pluripotency induction can be divided into virus-mediated and virus-free (Table 1).

Lentiviruses and retroviruses are vectors that can integrate randomly into the genome of cells and might disrupt active genes or regulatory regions. Such genomic insertions can activate endogenous oncogenes *via* knock-out of some genes, e. g. oncogene repressor, and lead to cancerogenesis (Baum *et al.*, 2004; Okita *et al.*, 2008). These vectors have been used to create iPSCs from adult human

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Abbreviations: Ac, acetylation; Dotl, disruptor of telomeric silencing-like; HMTs, histone methyltransferases; iPSCs, induced pluripotent stem cells; Me, methylation; ncRNAs, noncoding RNAs; SeV, Sendai viruses

Table 1. Summary of the iPSCs strategies
Table includes established methods for iPSCs derivation. They involve viral and nonviral approaches with their advantages and disadvantages.

Reprogramming methods			Advantages	Disadvantages
viral	integrating vectors	lentiviruses	- high efficiency, - infecting of nondividing and proliferating cells	- potential immunogenicity, - risk of active gene / regulatory region disruption, - risk of endogenous oncogene activation
		retroviruses	- high efficiency, - simplicity, - economy	- potential mutagenicity, - tumorigenicity, - low efficiency, - risk of active gene / regulatory region disruption, - risk of endogenous oncogene activation
	nonintegrating vectors	adenoviruses	- no integration into the host genome	- very low efficiency
		Sendai viruses	- easily removable, - a higher efficiency than retrovirus	- high costs
	transgene excision	Cre-loxP system	- can infect nondividing and proliferating cells	- risk of insertional mutations, - harmful genetic alterations
		piggyBac transposon system	- quite high efficiency (0.02–0.05%)	- harmful genetic alterations, - no published data that vector could be cleanly excised from the iPSCs, - labor-intensive
nonviral	with nucleic acid	DNA plasmids	- integration into the genome is not required	- repeated transfections, - low efficiency
		episomal vector system	- integration into the genome is not required	- repeated application, - low efficiency
		minicircles	- longer permanent transgene expression, - free of foreign or chemical elements	- low efficiency
		liposomal magnetofection	- simplicity, - short reprogramming times (8 days or less)	- potential toxicity
		synthetic RNA	- quicker and higher efficiency than standard viral techniques	- labor-intensive
		miRNA	- high efficiency	- risk of nonspecific, off-target effects, - instability
		nucleic acid derivatives	- effortless synthesis, administration and standardization, - cost-effective and simple storage requirements, - nonimmunogenic	- potential tumorigenicity, - no true specificity
	without nucleic acids	recombined proteins	- skipping genetic modification	- low efficiency - effective only in the fibroblast cell type
		small molecules	- high efficiency	- potential tumorigenicity - no true specificity

cells – in a retroviral system, the cells were transduced with Oct4, Sox2, Klf4 and c-Myc factors (Takahashi *et al*., 2007) and in a lentiviral – with Oct3/4, Sox2, Nanog, Lin28 (Yu *et al*., 2007). To deal with incorporation of viral vector sequences into the iPSC genome, alternative reprogramming systems using non-integrating adenoviruses have been developed, however a significant weakness of these systems is very low efficiency (Stadtfeld *et al*., 2008; Zhou & Freed, 2009). A better efficiency of pluripotent stem cells induction might be obtained *via* a system using episomal plasmids delivered by non-integrating Sendai viruses (SeV), where the RNA virus can be easily removed with antibodies, though the cost of this method is much higher than of the other viral methods (Fusaki *et al*., 2009; Sachamitr *et al*., 2014).

There are two systems facilitating the removal of genes integrated with the mouse genome or human iPSCs – the Cre-loxP and PiggyBac transposon systems (Zhou & Zeng, 2013). The first consists of a single viral vector equipped with a cassette of four transcription factors which are flanked by the loxP sites. The Cre-recombinase is delivered to the cell's nucleus by using the *Pseudomonas aeruginosa* bacteria and then overexpressed. Cre-mediated recombination leads to excision of the DNA sequences between the two loxP repeats (Kaji *et al*., 2009; Soldner *et al*., 2009). Another system is based on a transient transposase activity. The reprogramming factors are cloned into a PiggyBac transposon. In the presence of a transiently expressed transposase, this vector can be integrated into the host genome and excised

from iPSCs after reprogramming (Kaji *et al.*, 2009; Woltjen *et al.*, 2009, Yusa *et al.*, 2009).

In the non-viral methods of reprogramming, DNA plasmids do not integrate into a genome but are maintained in a cell for a few cell cycles and transiently express reprogramming factors (Okita *et al.*, 2008; Stadtfeld *et al.*, 2008; Yu *et al.*, 2009). An episomal vector system, in turn, is based on the Epstein-Barr Nuclear Antigen-1 that undergoes a permanent extrachromosomal replication in synchrony with the host genome, i.e. only once per cell cycle (Yu *et al.*, 2009; Okita *et al.*, 2011). Another system based on an episomal DNA vector, the minicircles, contains only cDNA of the expressed Yamanaka factors and a eukaryotic promoter (Jia *et al.*, 2010). A self-assembly of complexes that consist of cationic lipids and plasmids or siRNA, with magnetic nanoparticles of iron, has been termed liposomal magnetofection (Mykhaylyk *et al.*, 2010; Park *et al.*, 2012). Such complexes require a magnetic field to transfect vectors into the cells. A different method of reprogramming uses synthetic mRNA encoding the Yamanaka factors, delivered into somatic cells *via* a cationic lipid vehicle. The mRNA is synthesized using *in vitro* transcription reactions, treated with modified ribonucleotides and a phosphatase, and the medium is supplemented with an interferon inhibitor which allows for lower cytotoxicity, acquiring high protein expression and improving cell viability (Yu *et al.*, 2007; Hanna *et al.*, 2009).

miRNA play a significant role in reprogramming through epigenetic regulation of chromatin remodeling complexes. Some miRNA clusters participate in control of genes related to maintenance of pluripotency (Subramanyam *et al.*, 2011). It has been demonstrated that miR93, as well as miRNA from the miR302 family, in combination with the Yamanaka factors, can enhance the efficiency of reprogramming (Li *et al.*, 2011; Subramanyam *et al.*, 2011). Furthermore, mir-200, mir-302 and mir-369 could induce pluripotency in human cells (Miyoshi *et al.*, 2011). A cocktail of miR 302-367 very quickly and efficiently reprograms the mouse and human somatic cells to the pluripotent state without additional reprogramming factors (Anokye-Danso *et al.*, 2011; Liao *et al.*, 2011). A genetic modification might be omitted by using methods that do not employ nucleic acids. Delivery of a recombined protein encoded by reprogramming factors into the cells, instead of these factors themselves, is one among those methods (Kim *et al.*, 2009).

Another nonviral method that allows avoiding genomic insertions and immunogenicity relies on utilization of small molecule compounds, including RNA-derivatives (Fig. 1). They may improve the quality of reprogramming, such as time and efficiency (Efe and Ding, 2011). It is worth to note that efficiency of reprogramming *via* such compounds highly depends on the specific cell type (Paull *et al.*, 2015). Because of low mass, which is limited up to 500 Da, they might diffuse freely across the cell membranes (Lipinski, 2004; Dougherty *et al.*, 2012). Given the easiness to synthesize, administer and standardize, as well as cost-effectiveness and simple storage requirements, small molecules are a promising approach to pluripotent cell induction (Hou *et al.*, 2013). However, this method displays some weaknesses, like potential tumorigenicity, mutagenicity, as well as possible targeting of endogenous cell components that are not specific to pluripotency.

INDUCERS OF PLURIPOTENCY

The fundamental mechanism of epigenetics is accommodation of gene expression in response to interactions between the genes and the environment (Morange, 2002). This can be highly manipulated in somatic cells and the cell identity may be reversed to the initial state of development or altered. Most of the small molecules are epigenetic modulators and influence methylation of DNA and histone modifications in the cells (Jaenisch, 2012). Methylation patterns of pluripotency gene promoters should be similar to those found in the embryonic stem cells (Maherali & Hochedlinger, 2009).

There are groups of compounds that are either proven or for now only putative inducers of pluripotency. 5-azacytidine and zebularine are cytidine analogues (Fig. 2) and act as DNA methyltransferase inhibitors. 5-azacytidine contains a nitrogen atom at position 5, whereas zebularine lacks the amino group at position 4 of the corresponding cytidine. It has been demonstrated that both compounds form covalent bonds with DNMT after incorporation into DNA (Taylor & Jones, 1982; Zhou *et al.*, 2002). 5-azacytidine, named also 5-AZ or AZA, may

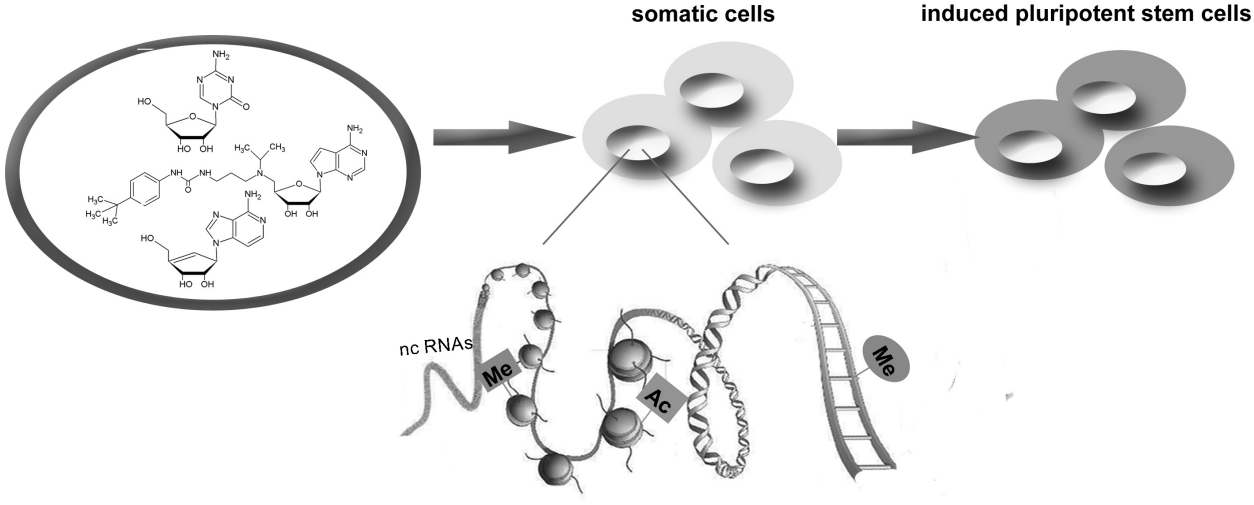

Figure 1. Schematic diagram of pluripotency induction *via* small molecules.
Small molecules cause cellular reprogramming through epigenetic changes, such as DNA methylation, histone modifications, noncoding RNAs and chromatin remodeling. Me, methylation; Ac, acetylation; ncRNAs, noncoding RNAs

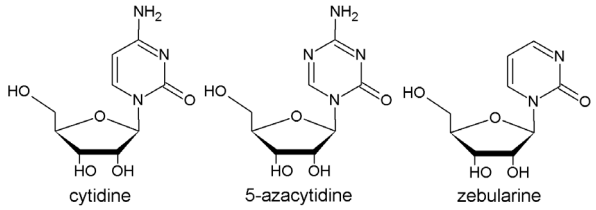

Figure 2. Chemical structures of cytidine and its analogues.

incorporate into both, DNA and RNA. 5-AZ is toxic and unstable under physiological conditions. When incorporated into nucleic acids *via* the sulfhydryl side chain of the catalytic cysteine residue, these compounds form a stable reaction intermediate. These nucleosides then become suicide substrates for the DNMT enzymes (Lyko & Brown, 2005). It is assumed that the vast majority of azacytidine is incorporated directly into the RNA and the rest (10–20%) is activated and converted by a ribonucleotide reductase into the active nucleotide for DNA methylation inhibition, 5-aza-2'-deoxycytidine-5'-triphosphate (Li *et al*., 1970; Stresemann & Lyko, 2008). 5-azacytidine can substitute for a cytosine, and azacytosine-guanine dinucleotides are formed which are recognized by the DNA methyltransferases as natural substrates (Stresemann & Lyko, 2008). As a result, a covalent bond between the carbon-6 of the cytosine and the enzyme is established (Santi *et al*., 1984; Chen *et al*., 1991). Substitution of carbon by the nitrogen atom at position 5 in azacytosine precludes the reaction of β-elimination through the carbon-5 atom, and thus DNMT remains covalently bound to DNA and its catalytic function is blocked. Furthermore, such covalent protein-DNA adduct triggers DNA damage signalling and trapped DNMTs are degraded, resulting in depletion of the cellular DNMTs and lost of methylation marks during DNA replication (Stresemann & Lyko, 2008).

5-azacytidine improves reprogramming efficiency by 3 folds (with an effective concentration of about 2 μM in mouse embryonic fibroblasts; MEFs) (Huangfu *et al*., 2008a; Mikkelsen *et al*., 2008). There are cases when some cells become trapped in partially reprogrammed states and show DNA hypermethylation at pluripotency-related loci. In such cases, 5-AZ enables to complete the iPSCs reprogramming (Huangfu *et al*., 2008a; Mikkelsen *et al*., 2008). Five μM concentration of 5-AZ boosts and may increase efficiency of reprogramming during late stages of this process in a doxycycline-inducible Oct4 expression screening system, in the presence of a cocktail that consist of valproic acid, CHIR99021, RepSox and tranylcypromine (Polo *et al*., 2012; Hou *et al*., 2013).

Besides its effects on reprogramming, 5-azacytidine has been also proved to participate in transdifferentiation events from one cell type to another. It participates in conversion of murine fibroblasts into adipocytes and bone cells, of mesenchymal stromal cells and fibroblasts into haematopoietic cells, of adult skin fibroblasts and granulose cells into highly permissive state and towards different cell lineages and phenotypes, of fibroblasts into insulin-secreting cells, of human granulosa cells into muscle cells with human recombinant vascular endothelial growth factor, and in transformation of adipose-derived stem cells into myoblasts (Taylor & Jones, 1979; Tamada *et al*., 2006; Pennarossa *et al*., 2013; Brevini *et al*., 2014; Wang *et al*., 2014).

Zebularine is a stable hydrophilic cytidine analogue with the depleted 4-amino group, and acts as a DNMT inhibitor and was formerly developed as a cytidine deaminase inhibitor (Zhou *et al*., 2002; Nakamura *et al*., 2013). It forms tight covalent complexes between the DNMT enzymes and DNA substituted with zebularine, which could lead to a compositional change in the DNMT protein, and thus it is conceivable that DNMTs can be then degraded *via* the ubiquitination system (Hurd *et al*., 1999; You & Park, 2012). Zebularine has been shown to exhibit low toxicity in mice (Cheng *et al*., 2003; Yoo *et al*., 2004, Cheng *et al*., 2004). This compound preferentially targets cancer cells (Andersen *et al*., 2010). It has been demonstrated that zebularine decreased the levels of DNMT1, DNMT3a, DNMT3b in cholangiocarcinoma, hepatocellular carcinoma cells bladder, cervical, and breast cancer cells (Cheng *et al*., 2004; Fandy, 2009; You & Park, 2012; Nakamura *et al*., 2013; Nakamura *et al*., 2015).

Zebularine is a proven inducer of pluripotency. It has been demonstrated to participate in reprogramming of the yak fibroblasts for cloning (Xiong *et al*., 2013).

Neplanocin A, 3-deazaneplanocin A, 3-deazaadenosine, D9 and EPZ004777 are adenosine analogues or derivatives (Fig. 3) and belong to proven and putative histone methyltransferase inhibitors.

Histone methyltransferases (HMTs) transfer methyl groups from the S-Adenosyl methionine (SAM) specifically onto either lysine or arginine residues of the H3 and H4 histones. There are two suggested mechanisms of the SAH hydrolase inhibition – either *via* oxidation of NAD^+ to NADH (type I – reversible), or *via* cova-

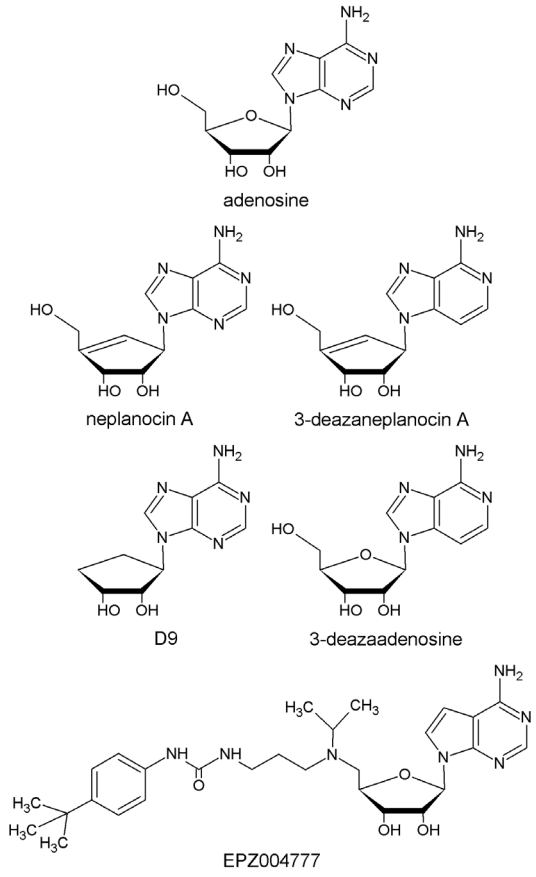

Figure 3. Chemical structures of adenosine and its analogues and derivative.

lent binding to the active site by an inhibitor with a nucleophilic residue (type II – irreversible) (Wolfe & Borchardt, 1991).

Naturally occurring neplanocin A, an analogue of adenosine with the oxygen atom substituted by carbon-5, and its derivative DZNep are effective inhibitors of the S-adenosylhomocysteine (SAH) hydrolase (Tam *et al*., 2015). However, both of these compounds are toxic, which is a result of phosphorylation of the C-5' primary hydroxyl group (Wolfe & Borchardt, 1991). Neplanocin A has been demonstrated to be metabolized *via* conversion into a 5'-triphosphate (Montgomery *et al*.; 1982, Saunders *et al*., 1985).

A neplanocin A analogue that lacks nitrogen at position 3, 3-deazaneplocin A (DZNep), acts as a SAH hydrolase inhibitor. It can productively deplete cellular levels of the EZH2 complex, effectively and selectively inhibit trimethylation of lysine 27 of histone H3 (H3K27me3) and lysine 20 of histone H4 (H4K20me3), and induce apoptosis in cancer cells (Chiang, 1998; Gordon *et al*., 2003; Tan *et al*., 2007). This compound has been shown to exhibit a minimal toxicity *in vivo* (Bray *et al*. 2000).

3-Deazaneplanocin A, as well as others such as: valproic acid, CHIR99021, RepSox, tranylcypromine, forskolin, TTNPB, have been used in order to induce factor-free reprogramming (Hou *et al*., 2013). At a concentration of 0.05–0.1 μM, DZNep, as well as a mixture of other small molecules, such as valproic acid, CHIR99021, RepSox, tranylcypromine and forskolin, facilitate up to 65 folds higher efficiency in reprogramming of MEFs (Hou *et al*., 2013). During late stages of this process, DZNep, in combination with valproic acid, CHIR99021, RepSox and tranylcypromine, boosts reprogramming in a DOX-inducible Oct4 expression screening system (Hou *et al*., 2013).

Through structure and activity relationship (SAR) analysis, as well as correlation of physicochemical properties, it has been identified D9, a neplanocin A analogue that lacks hydroxymethyl group at position 4'. As an analogue of DZNep, it shows a comparable cellular activity with DZNep, about 20 fold less toxicity in mice and could potentially affect reprogramming (Jiang *et al*., 2015; Tam *et al*., 2015). D9 has been reported to induce suppression of histone methylation marks, such as H3K27me3 and H4K20me3, and to a lesser extent on H3K4me3 and H3K79me2, and had only little effects on H3K9me2 and H3K9me3 (Jiang *et al*., 2015).

3-Deazaadenosine (DZA) is an adenosine analogue lacking the nitrogen atom at position 3 and also acts as a SAH hydrolase inhibitor and leads to a rapid loss of H3K4 trimethylation in the ESC, followed by ESCs differentiation and death (Shyh-Chang *et al*., 2013). DZA, in the presence of valproic acid, CHIR99021, RepSox, tranylcypromine and forskolin participates in the reprogramming induction (Hou *et al*., 2013).

Inhibition of the catalytic activity of the H3K79 histone methyltranferase (Dotl, disruptor of telomeric silencing-like) is key to reprogramming. Mono-, di-, and trimethylation of H3K79 are all entirely catalyzed by Dot1l (Nguyen & Zhang, 2011). EPZ004777 is a 7-dezaze with added urea and phenyl fragments. This small molecule inhibits Dot1l which is followed by a decrease in the H3K79me2 levels, at concentrations ranging from 1 μM to 10 μM (Onder *et al*., 2012), and affects the iPSC reprogramming (Lin *et al*., 2009). By using EPZ004777 in mouse and human fibroblasts, the yields of four transcription factors-mediated induction of pluripotency increased by 3–4 folds (Onder *et al*., 2012). The iPSCs generated through the Dot1l inhibition show all the hallmarks of pluripotency. They have exhibited characteristic ESC morphology, have differentiated into all three germ layers *in vitro,* as well as in teratomas. The Dot1l inhibition substitutes for Klf4 and c-Myc (Onder *et al*., 2012).

PERSPECTIVES

The potential of nucleic acid derivatives to develop medical treatment of degenerative diseases and advance the field of regenerative medicine should profoundly increase in the near future. Such compounds may target specific signaling pathways and mechanisms and trigger pluripotent stem cells induction, thus they are effective tools for cell manipulation and development of therapeutic approaches for regenerative medicine (Ma *et al*., 2013, Chin *et al*., 2009; Nie *et al*., 2012; Hou *et al*., 2013; Jung *et al*., 2014). Usage of small molecules may lead to development of cell-based therapies and modelling of diseases *via* the production of patient-specific stem cells (Tang *et al*., 2016). Because reprogramming efficiency *in vitro* depends on the specific donor cell type and culture conditions, an appropriate usage of their combinations under proper conditions is needed.

Revealing and studying the influence of new nucleic acid derivative compounds on reprogramming is riveting and might lead to understanding the mechanisms underlying their activity.

Recently, there was a big progress in the small molecules application, however, many limitations that do not allow the use of such compounds in clinical settings in a large scale still remain. Modifications of particular structural sites or substitutes in derivatives of nucleic acids or other natural compounds influence the modulating activities of these small molecules, especially their inhibiting activity. Further pharmacological studies will provide data allowing identifying the optimal pluripotency induction conditions. Molecular mechanisms underlying the activity of small molecule compounds need to be fully elucidated. Insight into the epigenetic changes during pluripotent stem cell induction and further chemical and pharmacological studies would improve understanding of the stem cell biology and the major mechanisms and pathways involved in the cell reprogramming, as well as support the development of potential therapeutic approaches (Bojarski, 2006; Frye, 2010).

Acknowledgements

This work was supported by the NCBR (National Centre for Research and Development, Poland) Strategmed program STRATEGMED1/233624/5/NCBR/2014 "Low molecular weight epigenetic modulators for activation of pluripotency of cells for regenerative medicine purposes" and by the Polish Ministry of Science and Higher Education, under the KNOW program.

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