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TET protein inhibitors: Potential and limitations

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ABSTRACT

TET proteins (methylcytosine dioxygenases) play an important role in the regulation of gene expression. Dysregulation of their activity is associated with many serious pathogenic states such as oncological diseases. Regulation of their activity by specific inhibitors could represent a promising therapeutic strategy. Therefore, this review describes various types of TET protein inhibitors in terms of their inhibitory mechanism and possible applicability. The potential and possible limitations of this approach are thoroughly discussed in the context of TET protein functionality in living systems. Furthermore, possible therapeutic strategies based on the inhibition of TET proteins are presented and evaluated, especially in the field of oncological diseases.

1. Introduction

Ten-eleven translocation methylcytosine dioxygenases (TET proteins) belong to iron(II)- and α-ketoglutarate-dependent dioxygenases. They (TET1, TET2 and TET3) catalyse successive oxygenation reactions in DNA (5-methylcytosine) [1,2]. TET proteins gradually convert 5-methylcytosine into 5-hydroxymethylcytosine, 5-formylcytosine, and finally 5-carboxycytosine. However, some high-impact studies suggest that TET proteins could also be involved in the oxidation of 5-methylcytosine in RNA [3–5]. The role of TET proteins in DNA demethylation is shown in Fig. 1. DNA cytosine modifications (5-methylcytosine, 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxycytosine) play a key role in the control of chromosome functions (e.g., the transcriptional profile of a cell, mediation of genomic imprinting, X-chromosome inactivation, and repetitive element repression). [6–8].

5-Methylcytosine (5mC; called the fifth base) is significantly involved in the repression of gene expression and transposable and repetitive elements in DNA. The human genome is methylated mainly on the CpG dinucleotide (~3% of all cytosine species)[11,12], and most CpG dinucleotides are methylated (60–80%) [13]. Nevertheless, short GC-rich, CpG-rich DNA sequences (CpG islands) that are predominantly unmethylated, have been found in DNA [14]. CpG dinucleotides are mostly involved in controlling gene expression. More than 70% of gene promoters show high CpG content [15]. Their hypermethylation strongly leads to the transcriptional silencing of responsive genes [16]. On the other hand, half of the CpG islands are distal to genes (so-called intergenic) or inside genes (so-called intragenic) [17]. An intragenic CpG island (its strong transcriptional activity) can serve as an alternative gene promoter, or stimulate the expression of the host gene. However, DNA methylation was also observed at sites other than CpG sequences [18]. In some cell types (human embryonic stem cells and brain tissue), its possible existence is also discussed [19,20].

Another cytosine species is 5-hydroxymethylcytosine (5hmC, also called the sixth base; 0.02–0.7% of all cytosine species) [11,12], stable

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epigenetic modification. Its frequency is sometimes lower than the 5mC frequency depending on the cell type (1–10% 5mC) [21]. In some of them, its abundance can be significantly higher depending on the cell type: ~10% in mouse embryonic stem cells [22] and up to 40% in Purkinje neurons [23]. Similar to 5mC, 5hmC plays an important role in the regulation of gene expression, but unlike 5mC, it is more associated with its activation [24].

The abundance of 5fC and 5caC is very low compared to other cytosine modifications (approximately 0.002–0.02% of all cytosine species) [11,12]. It is possible that they do not only intermediate in DNA demethylation within the regulation of gene expression or repair mechanisms [25–28]. But they, especially 5fC, can also serve as epigenetic marks in DNA. For example, 5fC and 5caC can suppress RNA polymerase II activity [28].

Based on numerous studies, dysregulation of TET proteins is significantly related to the pathogenesis of many diseases [29–32]. This review is focused on inhibitors of TET proteins, which would regulate the activity of TET proteins and could have significant therapeutic potential. In the review, the activity of TET protein is discussed in the context of 5mC and 5hmC levels, since the level of 5fC and 5caC is several orders of magnitude lower and commonly used methods cannot be used for their determination [33].

2. Structure and properties of TET proteins

In humans, three TET protein isoforms encoded by different genes (*TET1*, *TET2*, and *TET3*) with similar structures and functions have been found (Fig. 2) [2,34]. Their catalytic domain is located in the C terminal region (double-stranded β-helix domain (DSBH)), a Cys-rich domain adjacent to the DSBH. The DSβH domain has three binding sites for the Fe(II) ions, each of which contains histidines and one aspartic acid residue) [35] and one for the α-ketoglutarate (α-KG; arginine) [35]. The low-complexity insert is used for the proteomic regulation of TET proteins. Both DSβH and Cys-rich domains form a conserved dioxygenase domain. Fu et al. reported that the catalytic domains of all three TET isoforms (TET-CD) can also oxidase 5mC to 5hmC in RNA [4]. Accordingly, it should be mentioned that reported inhibitor activity and other values are usually determined for the catalytic domain of TET proteins (Table 1).

Some TET1 and TET3 protein isoforms contain two Cys4-type zinc fingers at their N-terminus (called CXXC domain) with specificity for the CpG dinucleotide sequence in gene promoters and CpG islands. The CXXC domains of TET1 and TET3 proteins can form a chromatin complex with the scaffold protein Sin3a and the histone acetyltransferase MOF and thereby suppress the acetylation of H4 histone at lysine 16 [36–39].

The human *TET1* gene expresses the full-length TET1 protein (TET1-FL), which prefers a methylated CpG sequence, and its short isoform (TET1s) without the recognition CXXC domain [40] Nevertheless, they still show CpG specificity, although it is significantly lower than in TET1-FL. However, the TET1 catalytic domain (Flag-HA-TET1-CD, 79 KD; model of TET1s) shows the conversion of 5fC to 5caC in RNA [3]. In a mouse model, TET1-FL was strongly preferred in the early embryos, embryonic stem cells (ESCs), and primordial germ cells (PGCs) [40]. On the other hand, TET1s shows ~10-fold higher expression levels than TET1-FL in most somatic tissues (e.g., heart, kidney, liver, muscle, spleen) [40]. In the mouse brain, TET1s was found to be predominantly expressed in neurons, whereas Tet1-FL is generally expressed at lower levels and more abundantly in glia [41]. Some high-impact studies strongly suggest that TET1s could play a significant role in some pathologies such as tumourigenesis and atherosclerosis [42,43].

The *TET2* gene expresses three isoforms (TET2-a, TET2-b, and TET2-c), but only TET2-b exhibits catalytic activity. TET2-a and TET2-b are mainly expressed in the spleen and normal tissues (placenta, spleen, bone marrow, embryos, lungs, and others), respectively [1,44,45] TET2-c is weakly expressed in most tissues, especially in the spleen, bone marrow, fetal brain, and embryoid bodies (3D-aggregates of pluripotent stem cells) [1,45]. Its selectivity is dependent on extrinsic factors such as Idax (CXXC Finger Protein 4; known as a repressor of Wnt signalling [46,47]). Its CXXC domain binds unmethylated CpG dinucleotide [48]. On the other hand, it was reported, that IDAX expression led to a decrease in TET2 protein through caspase activation in HEK293T, and shRNA against IDAX increased TET2 protein expression in the human monocytic cell line U937. However, in the Idax$^{-/-}$ NPC cells, a decrease in *TET1* and *TET2 mRNA* and an increase in *TET3 mRNA* versus wild-type cells were observed [49].

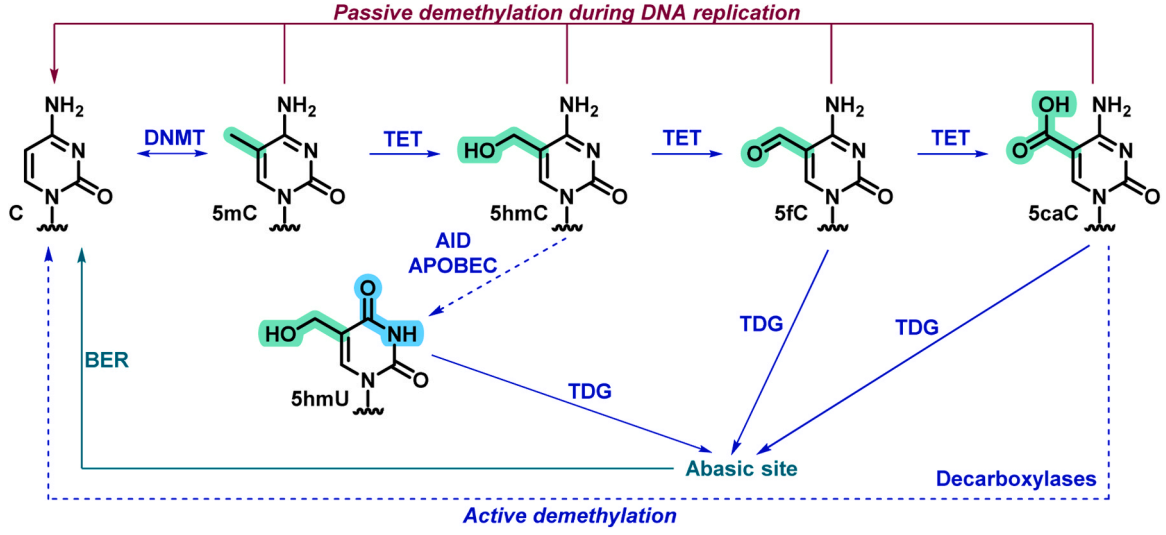

Fig. 1. The role of TET proteins in DNA demethylation [9]. Cytosine (mostly in the CpG dinucleotide) is methylated at the 5th position by DNA methyltransferase (DNMT1, DNMT3A, and DNMT3B). 5mC is gradually oxidised on 5hmC, 5fC and 5caC. It should be noted that 5mC can be demethylated by DNMT3A and DNMT3B [10]. It is assumed that 5caC can be decarboxylated to original cytosine by decarboxylases. 5fC and 5caC can be also excised by TDG. 5hmC could be recognized and oxidized by AID APOBEC. The formed abasic site is repaired by BER and results in the restoration of the unmodified cytosine state. These enzymatic mechanisms are called active demethylation. Passive demethylation can be observed in DNA replication, the newly replicated DNA strand cannot contain 5mC as the original DNA. [9] **Abbreviations:** 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; 5fC, 5-formylcytosine; 5caC, 5-carboxycytosine; APOBEC, apolipoprotein B mRNA editing enzyme; DNMT, DNA methyltransferase; C, cytosine; TDG, thymine DNA glycosylase; TET, ten-eleven translocation.

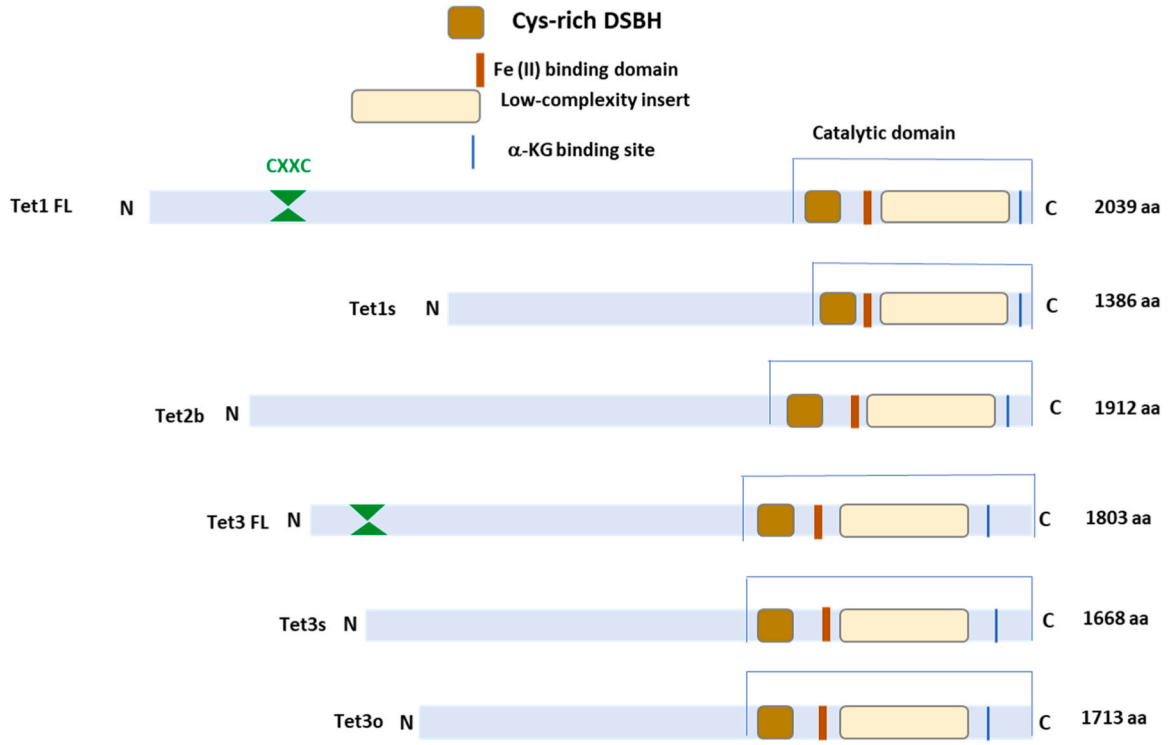

Fig. 2. TET protein isoforms.[1,2] TET proteins are encoded by three independent genes that have several isoforms. Each of them contains a catalytic domain (called also dioxygenase domain in the C terminal region), which consists of an a-KG binding site, low-complexity insert (proteomic regulation of enzyme), Fe(II) binding domain, and Cys-rich double-stranded β-helix domain. Some of them (TET1 FL and TET3 FL) contains also the CXXC domain (in N terminal region) with the specific CpG dinucleotide sequence. The selectivity of TET protein isoforms without the CXXC domain (TET1s, TET2b, TET3s, and TET3o) is controlled by extrinsic factors such as Idax. TET2a and TET2c display no catalytic activity and therefore are not presented in the figure. In the figure, the mouse isoforms of Tet proteins are shown. The structure of the human isoforms is similar, but with a different number of amino acids: TET1 FL (2.136 aa), TET1s (1465 aa.), TET (2002 aa.), TET3 FL (1795 aa.), TET3s (1660 aa.). Human TET-3o has not been reported.

The *TET3* gene expresses three isoforms (TET-FL, TET3s, and pTET3L). TET3-FL (predominant form expressed in neurons) shows a strong preference for CpGs modified by 5caC, slower for unmethylated CpGs [50–52]. In this context, it is interesting to note that TET3-FL, unlike TET1-FL and the full-length TET2 protein, shows the oxidation of 5mC to 5hmC in RNA [4]. TET3s was also observed in neuronal cells, whose hydroxylase activity was strongly increased by other factors such as the transcriptional regulator REST [53]. However, in mammalian neuronal cells, TET3 protein expression did not cause demethylation in CpGs but increased intragenic 5hmC [51]. An oocyte-specific TET (TET3o) was found as a unique oocyte-specific isoform and the dominant transcript of TET3 protein with the CXXC domain in pig embryos [54]. Tet3o was observed in a mouse model but without the CXXC domain [50]. Nevertheless, in mouse zygotes, TET3 protein depletion did not cause a decrease in the 5hmC level [55].

3. Specificity of the mitochondrial epigenome

A significant majority of studies about TET proteins, including their inhibitors, are primary focused on the regulation of nuclear DNA. It is true that they have significantly contributed to a better and deeper understanding of the epigenetic mechanisms in living systems. However, the role of mitochondrial DNA should not be neglected. It can be expected that a better and more detailed knowledge of mitochondrial DNA regulation, including the role of the TET proteins, can significantly reduce the limitations in the understanding of mitochondrial behaviour and functionality and the applicability of TET proteins inhibitors. In this sub-chapter, the epigenetic regulation of mitochondrial DNA is presented with an emphasis on the possible role of TET proteins.

In addition to nuclear DNA, eukaryotic cells also contain mitochondrial DNA (mtDNA; Fig. 3). Unlike nuclear DNA (nDNA), mtDNA is circular and significantly smaller (16,569 vs. 3.3 billion bp) [56]. mtDNA encodes 22 transfer and 2 ribosomal RNAs and 13 polypeptides (part of oxidative phosphorylation complexes). Other mitochondrial proteins are encoded by nDNA [57]. 28 mitochondrial genes and the upper part of the displacement loop (D-loop) are located on the heavy strand, while the light strand contains only 9 genes [58,59]. The D-loop is a non-coding region (nearly 600 bp), containing the origin of replication (ori H), the heavy strand promoters (I_{H1} and I_{H2}) located on the heavy strands, and the light strand promoter (I_L).

It is well known that mtDNA disturbances are deeply associated with the pathogenesis of many serious diseases such as oncological, metabolomic, and neurodegenerative diseases [60–63]. Unfortunately, compared to the nuclear epigenome, our knowledge of mitochondrial epigenetic mechanisms is very fragmented and limited [64,65]. However, thanks to enormous efforts, we can take a look behind the curtain. The existence of 5mC in the mtDNA has been recently confirmed [66, 67]. However, the frequency of 5mC in mtDNA was significantly higher than that of the corresponding nDNA [66,67]. Nevertheless, CpG methylation in mtDNA was low (3–5%) compared to nDNA [68]. Analysis of methylated cytosines implies that most of them were located outside CpG nucleotides in the L strand (D-loop) [69] and showed high dynamics, for example, in fibroblasts. A decrease was observed as the age of the culture increased [70].

The presence of 5hmC has also been reported in mtDNA [71–73]. Hydroxymethylation in mtDNA CG (~0.6% cytosine context) was comparable to nDNA but was more than an order of magnitude greater at other CH sites (H=A/C/T; ~0.5%) [73]. A detailed analysis, published by Ghosh et al. showed that 5mhC is localized near the gene start sites (GSS) rather than in the coding regions of mitochondria-encoded genes. However, the genome-wide pattern of 5hmC in mtDNA is not conserved and rather dynamic compared to methylcytosine [74].

Table 1
Inhibitory activities of low-molecular and peptide inhibitors of TET proteins.

Inhibitor	TET protein[a]	Inhibitory activity	Conditions	Ref.
Ni(II)	TET1	IC_{50} = 1.2 μM	UHPLC-MS/MS assays (1 mM ascorbate, 1 mM α-KG, 10 μM $FeCl_2$, pH = 8)	[87]
Cd(II)	TET1	84% at 2.0 μM		
Succinate	TET1	IC_{50} = 540 μM	HPLC-MS (5 mM ascorbate, 0.6 mM 2-oxo[1-^{14}C]glutarate and 0.05 mM $FeSO_4$, pH = 7.8)	[77]
	TET2	IC_{50} = 570 μM		
Fumarate	TET1	IC_{50} = 390 μM		
	TET2	IC_{50} = 400 μM		
2-HG	TET1	72% at 10 mM 53% at 50 mM	Dot blot (2 mM ascorbate, 0.1 mM of α-KG, 75 μM, Fe $(NH_4)_2(SO_4)_2$, pH = 8)	[99]
	TET2	67% at 10 mM 27% at 50 mM		
Inhibitor **1**	TET1	IC_{50} = 1.5 μM	ELISA Assay (SIGMA A8706; 0.1 mM ascorbate, 10 μM α-KG and 25 μM $Fe(NH_4)_2(SO_4)_2$, pH 6.5)	[92]
	TET2	IC_{50} = 9.4 μM		
	TET3	IC_{50} = 8.8 μM		
Inhibitor **2**	TET2	IC_{50} = 11.3 mM	orthogonal MALDI assay (2 mM ascorbate, 1 mM α-KG and 75 μM $Fe(NH_4)_2(SO_4)_2$, pH = 7.0)	[103]
Inhibitor **3**	TET2	IC_{50} = 46 μM		
Inhibitor **4**	TET1	IC_{50} = 1.0 μM	ELISA (Sigma-Aldrich, 25 α-KG A8706 50 μM $Fe(NH_4)_2(SO_4)_2$, pH = 6.5)	[104]
	TET2	IC_{50} = 1.3 μM		
	TET3	IC_{50} = 1.8 μM		
Inhibitor **5**	TET1	IC_{50} = 0.79 μM	Abcam TET Hydroxylase Activity Quantification Kit (2 mM ascorbic acid, 1 mM α-KG 0.1 and 1 mM $FeSO_4$)	[105]
Inhibitor **6**		IC_{50} = 9.1 μM		
Inhibitor **7**		IC_{50} = 8.0 μM		
Inhibitor **8**		IC_{50} = 15.0 μM		
Inhibitor **9**		IC_{50} = 1.3 μM		[106]
Inhibitor **10**		IC_{50} = 4.8 μM		[107]
Inhibitor **11**		IC_{50} = 6.27 μM		
Inhibitor **12**	TET1	IC_{50} = 3.48 μM	Dot blot (mM L-Ascorbic Acid, 1 mM α-KG and 100 μM $(NH_4)_2Fe(SO_4)_2$, pH = 8)	[108]
	TET2	IC_{50} = 1.2 μM		
	TET3	IC_{50} = 2.31 μM		
Inhibitor **13**	TET1	IC_{50} = 0.72 μM	AlphaScreen technology (100 μM ascorbate, 10 μM α-KG 10 μM and 10 μM $(NH_4)_2Fe(SO_4)_2$, pH = 6.7)	[109]
	TET2	IC_{50} = 1.10 μM		
Inhibitor **14**	TET2	IC_{50} = 2.3 μM	orthogonal MALDI assay (2 mM ascorbate, 1 mM α-KG, 75 μM $Fe(NH_4)_2(SO_4)2$, pH 7.0)	[103]
Inhibitor **15**	TET2	IC_{50} = 23.9 μM		
Inhibitor **16**	TET1	IC_{50} = 33 μM	Chemiluminescence ELISA (Bioscience; 50651; 0.1 mM α-KG and 0.12 mM $(NH_4)_2Fe(SO_4)_2$)	[110]
	TET2	IC_{50} = 73 μM	Chemiluminescence ELISA (Bioscience; 50652; 0.1 mM α-KG and 0.004 mM $(NH_4)_2Fe(SO_4)_2$)	
Inhibitor **17**	TET1	IC_{50} = 1.481 μM	AlphaScreen technology (100 μM ascorbate, 10 μM α-KG 10 μM and 10 μM $(NH_4)_2Fe(SO_4)_2$, pH = 6.7)	[109]
	TET2	IC_{50} > 10 μM		
Inhibitor **18**	TET1	IC_{50} = 1.126 μM		
	TET2	IC_{50} = 2.420 μM		
Inhibitor **19**	TET1	IC_{50} = 1.435 μM		
	TET2	IC_{50} = 1.620 μM		

b2-hydroxyglutarate
[a] The compact catalytic domain of the TET proteins was used to determine the inhibitory activity.

Dzitoyeva et al. reported that Purkinje cell samples from old mice (24 months) showed higher 5hmC (non-CpG recognition sites; AGCT, CCTC, and GGCC) and *Tet mRNA* levels than samples from young mice (4 months); *Tet1 and Tet2 mRNA* levels were not significantly different [72]. However, Bellizzi et al. observed TET1 and TET2 in the mitochondrial fraction of HeLa cells. On the other hand, only Tet1 was found in the case of 3T3-L1 cells [69]. Similarly, Chen reported that valproic acid (a histone deacetylase inhibitor) decreased the Tet1 protein level in mitochondria associated with a reduction of 5hmC in the mtDNA in the 3T3-L1 cells [75].

4. Regulation of TET proteins with an emphasis on inhibition of enzyme activity

Given the importance of TET proteins in cells and the body, their mutations and dysregulations can play a significant role in the pathogenesis of many serious diseases, for example, oncological and neurological ones. At the molecular level, the activity of TET proteins is directly controlled by the levels of Fe(II), α-KG, O_2 (TET cofactors and substrate), and vitamin C, which strongly influence the activity of TET proteins (Fig. 4). And it should not be omitted, the activity of TET proteins is also regulated by numerous posttranslational modifications. [1].

Decreasing the level of intracellular oxygen level could be an effective way to inhibit the activity of TET proteins. For example, in embryonic stem cells, the oxygen level is reduced, and the level of the DNA hydroxymethylation-catalyzed Tet1 protein strongly decreases [76]. In the case of the TET2 protein, the effect was significantly slower, however, a decrease in oxygen content causes a strong increase in the level of DNA hydroxymethylation. The Km value of Tet1 and Tet2 for O_2 is 30 μm [77]. It could be suggested that this effect is probably not directly related to the repression of enzyme activity, but to the regulation of enzyme expression.

Another possible strategy could be to target iron levels. In cells, the concentration of labile iron (mainly Fe(II) ions) is usually in microsomal ranges [78,79]. For example, in liver endothelial cells, the determined values were: 16.0 ± 4.0 μM in the lysosome, 7.3 ± 2.6 μM in the cytosol, 11.8 ± 3.9 μM in nuclei, and 9.2 ± 2.7 μM in mitochondria [80]. The Km values of TET proteins (4.8 and 3.6 μM for the TET1 and TET2 protein, respectively) for Fe(II) ions suggest that a decrease in iron level could lead to a decrease in TET protein activity and 5hmC level [77]. Iron deficiency in Sprague-Dawley rats reduces Tet activity in the P15 cerebellum but with minimal effect on DNA hydroxymethylation [81]. However, the P15 hippocampus was found to increase DNA hydroxymethylation, TET protein activity, and TET3 protein expression. Similarly, Dickson et al. reported that in the presence of ascorbate (10 μM), 5hmC generation was independent of the iron supplement (0–2.5 μM) and weakly dependent on glucose supplement (0–25 mM;

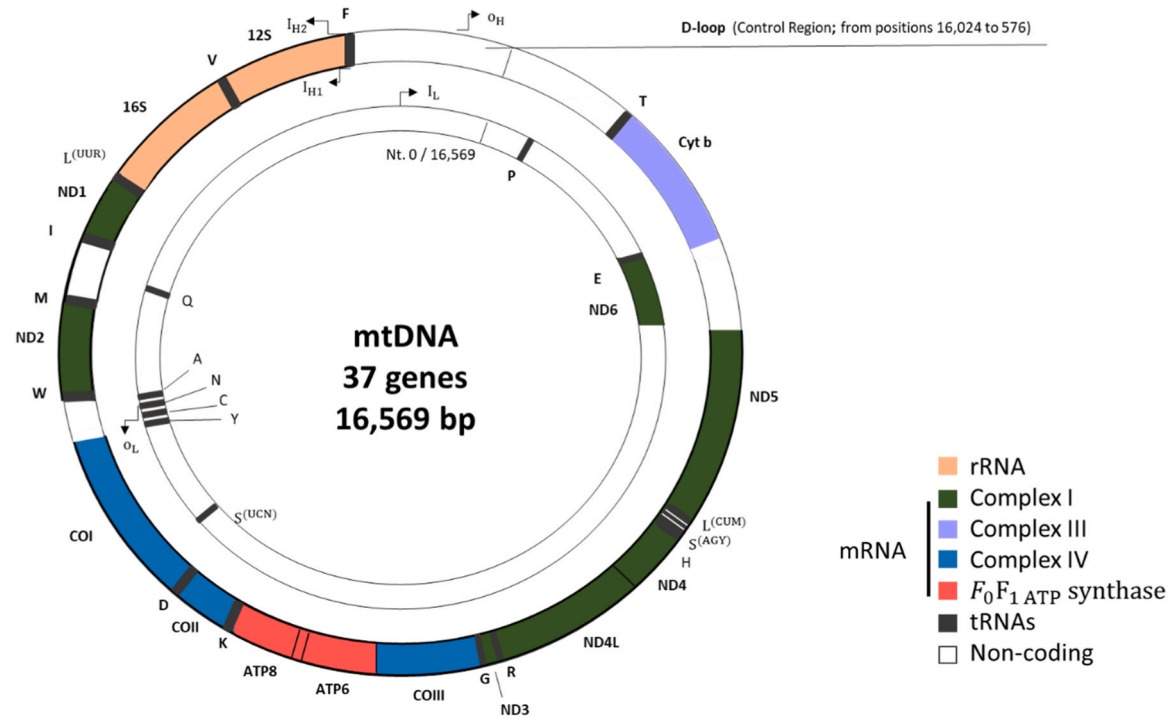

Fig. 3. Human mitochondrial DNA structure. mtDNA consists of heavy and light strands. The majority of mitochondrial genes are localized in heavy strain. mtDNA encodes 2 ribosomal RNAs (12 S and 16D) tRNAs (named after the single-letter abbreviation of the corresponding amino acid) and 13 polypeptides. Encoded polypeptides are parts of oxidative phosphorylation complexes: complex I (ND1, ND2, ND3, ND4L, ND4, ND5, and ND6), complex III (cytochrome) and complex IV (COI, COII, and COIII) and ATP synthase (called also Complex V; ATP6 and ATP8). mtDNA contains non-coding sequences such as D-loop, which control mtDNA transcription (I_{H1} and I_{H2}, heavy strand promoters and IL, light strand promoter) and replication (origin for H-strand replication, O_H).

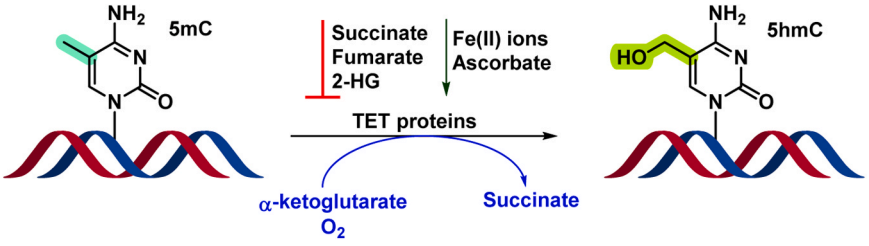

Fig. 4. Influence of intracellular metabolites on the activity of TET proteins. TET proteins are iron(II)- and α-ketoglutarate-dependent dioxygenases, therefore their substrates (α-KG and O_2) and cofactor Fe(II) ion stimulate their catalytic activity. Ascorbate stimulates their enzymatic activity by regeneration of oxidized Fe(III) to Fe(II) ions. On the other hand, produced succinate and other products of mitochondrial metabolism (fumarate and 2-hydroxyglutarate) can compete with α-KG binding site and thereby repress enzyme activity.

stimulation of α-KG production) in mouse embryonic fibroblasts [82]. Ascorbic acid can directly interact with TET proteins and recycle used Fe (III) to Fe(II) ions. In the physiological iron concentration (10 μM), it sometimes increases the activity of TET proteins (Tet1 CD and Tet2 CD) [83]. However, with an excess of iron (100 μM), this effect may be absent [22].

It is true that increasing the level of intracellular labile Fe(II) by cAMP can stimulate the formation of 5hmC [84]. However, any reduction in the intracellular level of Fe(II) ions may not always suppress the activity of TET proteins and DNA hydroxymethylation. In a rat model, the application of iron suppressed the activity of succinate dehydrogenase, and the higher level of succinate reduced the activity of the Tet protein and mitochondrial level of hydroxymethylcytosine [85]. On the other hand, CBD (a potent iron chelator) suppresses the effects induced by iron and thus restores mitochondrial DNA hydroxymethylation. In ovarian granulosa cells, the application of ferrostatin-1 (an inhibitor of ferroptosis) leads to an alleviation of homocysteine-induced injury, most probably caused by increased activity of TET proteins [86]. However, the intracellular level of Fe(II) ions was strongly decreased. Nevertheless, a high dose of deferoxamine (1 mM) suppresses the activity of TET proteins in the mouse zygote [55].

On the other hand, bivalent ions, which ideally have a similar ionic radius to the Fe (II) ion (ionic radius = 0.061 nm), can occupy the Fe binding site of TET proteins [87]. For example, Ni(II) ion (ionic radius = 0.069 nm) shows strong inhibitory activity against TET1 protein (IC_{50} = 1.2 μM). The inhibitory activity of the Cd(II) ion (ionic radius = 0.095 nm) was significantly lower.

The above strongly suggests that the regulation of TET proteins in biological systems is a complex sophisticated system and the utility of targeting it in controlling the activity of TET proteins may be limited. On the other hand, specific inhibitors of TET proteins could represent more effective tools for these purposes. And their widespread application could significantly contribute to a deeper understanding of this phenomenon.

5. TET protein inhibitors

It is also well known that mitochondrial metabolites (e.g., 2-oxoglutarate, succinate, fumarate, and citrate) can control the degree of DNA methylation and histone modification (e.g., acetylation and methylation) [88]. Lower levels of fumarate and succinate (inhibitors of TET proteins; IC_{50} values ~0.4–0.5 mM) [77] have been found in patients with Alzheimer's disease (AD) [89,90]. Ellison et al. reported that higher levels of 5hmC could be observed in the brain of AD patients and were

significantly elevated in subjects with preclinical Alzheimer's disease (PCAD) and mild cognitive impairment (MCI) compared to controls, although were dependent on brain region and disease stage [91]. Cancers with succinate dehydrogenase and fumarate hydratase mutations can show high accumulation of succinate and fumarate (micromolar concentrations) [77]. However, fumarate and succinate can modulate the expression of TET proteins [77]. In neuroblastoma cells, *TET1* and *TET2 mRNA* levels were down-regulated, while *TET3 mRNA* was up-regulated by fumarate but not succinate. On the other hand, hypoxia decreases the expression of TET2 and TET3 proteins and increases the

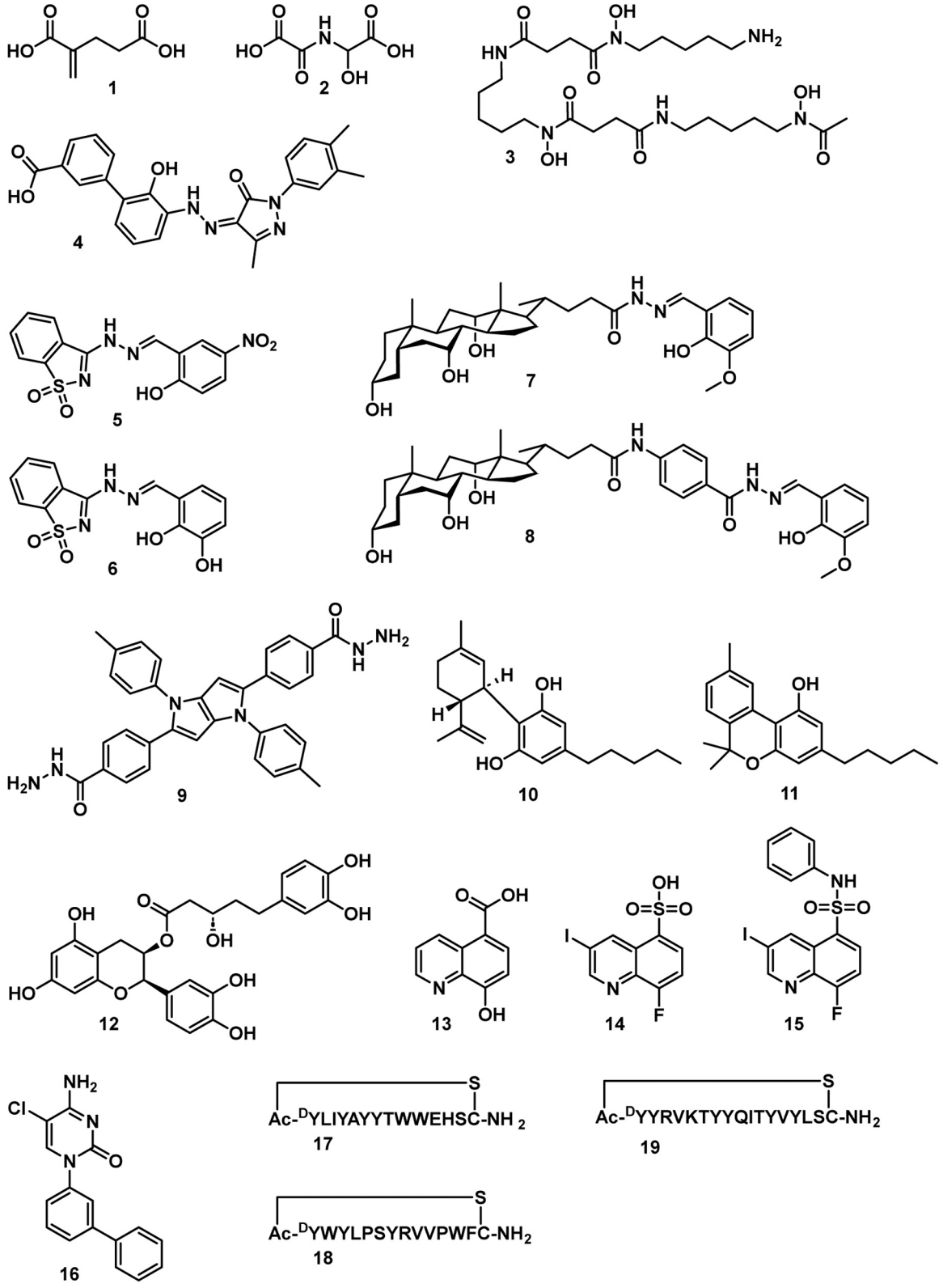

Fig. 5. Low-molecular (**1**-**16**) and peptide inhibitors (**17**-**19**) of TET proteins. Low-molecular inhibitors can be divided into carboxylic acid derivatives (**1** and **2**), iron chelators (**3**-**12**), and nucleobase analogs (**13**-**16**).

TET1 protein [77]. In some types of tumours (e.g., acute myeloid leukaemia), mutation of TET proteins, especially TET2 protein, should be considered in anticancer treatment. Laukka et al. reported that the H1302Y and D1304A mutations of TET2 protein increased the Km values for Fe(II) ions and 2-oxoglutarate by 30–80 times and decreased the V_{max} values. It cannot be excluded that an increase in the local concentration of iron or 2-oxoglutarate in the bone marrow could at least partially restore their activity and even reverse their oncogenic properties. According to the proposed hypothesis, TET protein inhibitors show promising therapeutic efficacy against TET2-deficient leukaemia in a mouse model [92]. However, in mice with $Tet2^{MT}$ myeloid neoplasia, ascorbic acid administration associated with increased TET protein activity resulted in prolonged survival of mice [93]. Nevertheless, vitamin C exhibits its own anticancer activity (ROS formation) independently of Tet protein activation [94].

Besides fumarate and succinate, the mitochondrial Krebs cycle can also produce 2-hydroxyglutarate (2-HG) [95–98]. In this context, it should be mentioned that 2-HG, fumarate and succinate also suppress the activity of other 2-oxoglutarate-dependent dioxygenases such as histone demethylases and HIF prolyl-4-hydroxylases with an effect on gene expression [98]. Some tumours (e.g., gliomas, chondrosarcoma, and acute myeloid leukaemia) with IDH1 and IDH2 mutations suppress α-KG production and stimulate 2-HG production (>35 mM) [95–97]. Xu et al. reported that 10 mM D-2-HG resulted in partial inhibition of TET1 and TET2 proteins (28% and 33%, respectively), while 50 mM 2-HG resulted in stronger inhibition (47% and 83%, respectively) [99]. Supplementation of ascorbic acid, however, leads to the reactivation of the enzymatic activity of TET proteins suppressed by 2-HG [100].

On the other hand, in uterine leiomyoma (the most common benign tumour of the female reproductive tract) [101], significantly higher levels of 5hmC were associated with up-regulation of *TET1* or *TET3 mRNA* compared to normal myometrial tissue [102]. In human uterine leiomyoma cells, the application of 2-HG (0.7 mM; 48 h) significantly decreases the level of 5hmC and cell proliferation. The same effect was observed in the case of TET1 and TET3 silencing by specific siRNAs [102].

The above experiences inspired us to design and study synthetic inhibitors of TET proteins. In terms of their molecular structure and functionality, they can be derived from carboxylic acid derivatives, an iron chelator, and a nucleobase analogue. Their structures are shown in Fig. 5 and determined inhibitory activity in Table 1. They are discussed and described in more detail in the following subsections.

5.1. Derivatives of carboxylic acids

Structural analogs of 2-oxoglutarate are nonspecific 2-OG-dependent dioxygenase inhibitors [111]. Similarly, 2-HG competes with the 2-oxoglutarate binding site. Some of these, such as dimethyloxalylglycine (DMOG) and N-oxalylglycine (NOG), are used in biological studies to inhibit TET proteins. However, their inhibitory activity is not very high. For example, in the case of NOG (compound **2**), the IC_{50} value for the TET2 protein was 11.3 mM [103]. Nevertheless, their application may provide relevant information on the role of TET proteins and methyl-oxidized DNA bases such as 5hmC, especially in embryogenesis. On the other hand, DMOG (1 mM) causes a significant increase in *TET3 mRNA* levels in bovine parthenogenetic embryos [112]. TET1 and TET2 protein expression represented by mRNA level was insignificantly compared to TET3 protein. DMOG-induced changes in the 5mC and 5hmC levels were associated with overexpression of the apoptosis-related gene BAX, while BCL-2 expression was decreased. However, in the cholangiocarcinoma cells, its antiproliferative effect was associated with decreased TET1 protein expression and DNA hydroxymethylation [113].

TETi76 (**compound 1**) shows strong inhibitory activity against TET proteins, especially TET1 protein (IC_{50} = 1.5 μM) [92,114]. In the case of TET2 and TET3 proteins, TETi76 inhibitory activity was sometimes slower, but still significant (IC_{50} = 9.4 and 8.8 μM, respectively) [92]. Its inhibitory activity was suppressed by α-ketoglutarate and practically independent of the Fe(II) level (25 and 250 μM). In leukemic K562 cells, its application leads to sensitization of leukemic cells to doxycycline. A higher effect was sometimes observed in the case of $TET2^{-/-}$ mutants [92]. Tumour reduction was sometimes observed in a mouse model treated with doxycycline [92].

5.2. Chelators

Since TET proteins contain Fe(II) ions in the enzyme active centre, targeting it with specific chelators could be an effective strategy. Deferoxamine (clinically used agent; **compound 3**) shows strong inhibitory activity against the TET2 protein (IC_{50} = 46 μM; 75 μM ammonium Fe(II) sulfate) [103]. The application of deferoxamine (1 mM) suppressed the activity of Tet proteins (mostly Tet3) and reduced 5hmC, but did not affect the level of 5mC in the mouse zygote [55]. In cholangiocarcinoma cells, deferoxamine (0.1 mM) reduced TET1 expression and the 5hmC level, this effect was associated with reduced cell viability [113]. At submillimolar concentrations, fluoroquinolones (norfloxacin, ciprofloxacin, and enrofloxacin) inhibited TET proteins in human embryonic kidney cells [115]. On the other hand, a significant part of their inhibitory effect (considering the concentrations used) is caused by the reduction of the level of free Fe(II) ions via chelation. Therefore, their application can lead to a decrease in the activity of other Fe(II)-dependent enzymes, such as Jumonji domain histone demethylases and collagen prolyl 4-hydroxylases with a strong effect on gene expression [115].

Eltrombopag (a non-peptidyl thrombopoietin receptor agonist; **compound 4**) is approved for the treatment of immune thrombocytopenia and periprocedural thrombocytopenia [116]. In addition, due to its strong iron chelating ability, it exhibits strong anti-inflammatory and proliferative effects and is an intensively studied as an agent for the treatment of cancer [117]. Guan et al. reported that Eltrombopag suppresses the activity of TET proteins (IC_{50} = 1.0, 1.3 and 1.8 for TET1, TET2 and TET3, respectively) [104]. The inhibitory effect (for TET2 protein) was not suppressed by the addition of Fe(II) ions (from 25 to 100 μM) but by the addition of Fe(III) ions (25 μM). Based on the observed results, the authors constructed an interesting model of the interaction of Eltrombopag with TET2 proteins. Eltrombopag cannot only interact with the Fe(II) ion in the TET protein, but its complex with the Fe(II) ion can also uptake the enzyme cavity. However, neither the Fe(III) ion nor the Fe(III) complex can uptake the cavity, and therefore Fe(III) ions can restore the enzyme activity. In mouse bone marrow mononuclear cells, Eltrombopag (1 μM) reduces 5hmC levels. Eltrombopag also reduces the monocytes and neutrophil counts in the bone marrow of transplanted mice. In this case, in TET protein-/- cells, this effect was significantly suppressed.

Hydrazones represent very effective and well-utilized structure motifs for chelators of metal ions [118,119]. Many of them showed interesting biological effects with promising potential in the treatment of neurodegenerative [120] and oncological diseases [121,122]. In this area, our research group prepared some compounds (mainly benzoisothiazole derivatives; **compounds 5** and **6**) with strong inhibitory activity against TET1 proteins [105]. As we expected, their inhibitory activity correlated with Fe(II) affinity (represented by the binding constant). Since the tested compounds are strong chelators, this effect can also be explained by a reduced amount of free Fe(II) ions. However, the concentration of Fe(II) in the test was 0.1 mmol/l and the concentration of chelators representing their IC_{50} could not have had a significant effect on the level of iron. On the other hand, under the following conditions, the chelators quickly form a complex with Fe(II) ions, and a number of chelators changed little depending on the amount of iron. It is believed that the Guan model proposed for the inhibitory activity of Eltrombopag could also be applied to other chelators with inhibitory activity against TET proteins. A promising structural motif for the design

of chelator inhibitors could be pyrrolo[3,2-*b*]pyrrole substituted with an iron-binding group (e.g., hydrazides), such as **compound 9**. The prepared inhibitor shows a strong affinity for Fe(II), strongly targeting the TET1 protein (IC_{50} = 1.33 μM; Kd = 27.2 μM) and exclusive mitochondrial localization (Pearson's correlation coefficient = 0.92) [106]. Pyrrolo[3,2-*b*]pyrrole derivatives with 2-hydroxy-benzylidene moieties did not show any significant inhibitory activity.

Singh et al. successfully designed and tested anthocyanidin derivative C35 (**compound 12**) as an inhibitor of TET2 protein (IC_{50} = 1.2 μM) [108]. Its effect on the TET1 and TET3 proteins was significantly less (IC_{50} = 3.48 and 2.31, respectively). In the HEK293T and MEF cells, its application (5 μM) sometimes decreases the level of 5hmC. In the case of HEK293T cells, the highest decrease was observed in CpG islands, whereas in MEF cells it was in the proximal promoter.

5.3. Nucleobase analogs

Since 5mC, 5hmC and 5 fc are the substrates of TET proteins, nucleoside analogs represent promising structural motifs for their inhibition. Palei et al. developed an inhibitor of the TET2 protein (IC_{50} = 2.3 μM) based on a quinoline structure motif with sulfonic acid moiety (**compound 13**) [103]. However, structure-activity relationships and molecular docking studies clearly indicate that the chelation of the Fe(II) ion in binding site of the enzyme is an essential part of its inhibitory activity. In this case, aminobenzene substitution (**compound 14**) increased the IC_{50} to 23.9 μM.

The cytosine analog Bobcat339 (**compound 15**) shows strong inhibitory activity against TET1 (IC_{50} = 33 μM) and TET2 (IC_{50} = 73 μM) proteins but its effect on DNMT3a is negligible [110]. In hippocampal neurons of HT-22, its application (10 μM, 24 h) reduces the level of 5hmC to one-third.

Its possible potential in anticancer treatment was reported by Bellavia et al. [123] In MCF-7 breast cancer, IL-1β induced a decrease in 5mC level and an increase in 5hmC level in genomic DNA (including IL-6 and IL-8 promotors), similar to azacytidine. On the other hand, the application of Bobcat339 reverts the effect of IL-1β on cytosine modification. Surprisingly, it was observed that a dose of 33 μM showed the same effect of change in DNA modification as a dose of 75 μM. According to the authors, TET2 protein was found to be constitutionally expressed independently of IL-1β or azacytidine, but TET1 protein was induced by both. At the proteomic level, Bobcat339 suppresses the upregulation of N-cadherin and Vimentin and downregulation of E-cadherin induced by IL-1β. In the wound healing assay, this agent strongly suppresses cell migration. In an in vitro bone metastasis model, Bobcat339 suppresses Raw264.7 invasiveness and the number of pits formed by mature osteoclasts.

6. Inhibition of TET proteins in anticancer treatment

Targeting epigenetic mechanisms represents a promising strategy for the treatment of many serious pathologies, including oncological diseases. In antitumour treatment, therapeutic methods are known for the effective elimination of primary tumours. However, therapeutic regimens to suppress metastases are still lacking. Nevertheless, some high-impact works have shown that TET proteins are important components of prometastatic signalling cascades.

Weinberg et al. reported that higher succinate and ablation of mitochondrial respiratory chain complex III cause a higher production of succinate and 2-HG and subsequently increase methylation [124]. For Treg cells, this effect was associated with a loss of their immunosuppressive function and, unfortunately, reduced viability in a mouse model. Phosphoserine phosphatase in melanoma (the most lethal form of skin cancer) [125,126] stimulates its metastatic activity [127]. Rawat et al. reported that its knockdown increased intracellular 2-HG level and decreased 5hmC level and increased histone H3K4me3 methylation (most likely due to inhibition of Jumonji histone demethylases) [127]. In mice, phosphoserine phosphatase repression sometimes leads to lower tumour growth and metastatic activity. The above shows that treatment of melanoma cells with 2-HG (0.1 mM; 72 h) leads to a significant decrease in their viability.

According to the above, the anticancer effect of Bobcat339, C35 and TETi76 could indicate the possible clinical applicability of this strategy [92,108,123]. Bai et al. found that cholangiocarcinoma tissues show high expression of TET1 and TET3 proteins [113]. In mice, knockout of the Tet1 protein reduces tumour volume and malignancy. The TET1 protein (or rather its short form) is overexpressed in acute lymphoblastic leukaemia. In the primary leukaemia cells, a decrease in TET1 expression (by the clinically used PARP inhibitor Olaparib) was associated with a decrease in the 5hmC level and impaired DNA repair and cell proliferation [128]. In a mouse model, Olaparib causes a visible reduction in the infiltration of human $CD3^+$ T-ALL cells in the liver and lungs. Nevertheless, the role of TET proteins in oncogenesis is not unambiguous and the choice of an appropriate therapeutic strategy can be complicated. Acute lymphoblastic leukaemia is also associated with down-expression of the TET2 protein, and its reexpression and stimulation of its activity (using 5-azacytidine and ascorbic acid, respectively) could also show promising therapeutic effects. [129].

On the other hand, a minimal level of TET-dioxygenase activity may be necessary to maintain cell viability, and targeting the activity of residual TET2 protein could be a promising therapeutic method [130]. However, some high-impact studies strongly suggest that loss of TET2 protein expression is one of the oncogenic mechanisms in breast and nasopharyngeal carcinoma [131,132]. In nasopharyngeal carcinoma cells (CNE1 and HNE1), down-expression of TET2 protein was associated with increased expression of TET1 and TET3 proteins [132]. In addition, its anti-oncogenic function was not associated with its enzymatic activity, but with its interaction with pyruvate kinase and subsequent repression of glycolysis.

However, Nishio et al. prepared unique thioether macrocyclic peptides with sometimes higher affinity for TET1CCD than normal TET1 and TET2 proteins [109]. A shorter version of TET proteins (called the TET compact catalytic domain), which lacks the CXXC DNA-binding domain, is overexpressed in breast, uterine, and ovarian cancers and is associated with poor prognosis [42]. In this context, it should be mentioned that hydrazine and hydrazide inhibitors of the TET1 protein were tested in the presence of ascorbic acid (2 mM). A negative correlation between TET1 protein and oncogenic mR-21–5p was observed in colorectal tumour tissue [133]. Decreased TET1 protein expression was associated with larger size and spread of the primary tumour and a higher risk of metastases in regional lymph nodes. Moreover, patients at advanced stage (IIIþ and IV) show significantly lower expression of TET1 than expression in patients at early stage (Iþ and II). Similarly, patients with endometrial cancer with higher TET1 protein expression show higher overall and progression-free survival [134]. Similarly, in a mouse model of lung cancer, overexpression of TET1 was associated with repression of metastatic activity [135].

Although cancer is associated with higher TET protein expression, the therapeutic importance of TET protein functionality cannot be linked to its enzymatic activity. Similarly, several times higher TET1 protein expression was detected in human lung tumour samples compared to control samples [136]. In addition, the advanced stage ($p = 0.021$) and current smoking status ($p = 0.0041$) were significantly associated. The expression of TET1 (a full-length protein) is most likely caused by a mutant form of p53 that cannot suppress its expression, unlike the wild type. In NSCLC cells, TET1 knockdown stimulates cellular senescence and genomic instability. However, cell lines with higher TET1 expression show lower levels of 5hmC. Nevertheless, H3K9 demethylation was reduced in TET1-depleted cells.

In the case of clinical trials, it should be considered that the expression and activity of the TET protein can be strongly influenced by the therapies used. For example, idarubicin (5 μM; topoisomerase II inhibitor) induces an increase in 5hmC and a decrease in 5mC DNA in

T47D cells (human breast carcinoma cells) [137]. This effect was most likely due to increased activity of the TET2 protein, independent of topoisomerase II activity. An increase in the level of 5hmC was also observed with other anthracyclines (doxorubicin, aclarubicin and daunorubicin). This could indicate the possible therapeutic potential of combining of anthracyclines with TET protein inhibitors in anticancer treatment. On the other hand, cancer patients and survivors sometimes show a higher risk of cardiovascular events [138,139]. In human cardiomyocytes, higher expression and demethylation activity of TET1 protein show a protective effect against ion channel dysfunction induced by the secretion of cancer cells or TGF-β [140]. Anthracyclines are well known to be cardiotoxic [141].

Briefly, the role of TET proteins in oncogenesis is not clear. Depending on the type of tumour and other conditions (e.g., the therapy used), TET proteins can be suppressor or oncogenic factors. Some high-impact studies suggest that in the case of melanoma or some leukaemias, inhibitors of TET proteins could represent promising therapeutic agents. Alternatively, a strategy could be to target some important mechanisms/parts of tumour development and metastatic spread, such as hypoxia and the cancer-immune system. The possible approach is discussed in more detail in the next subsections.

6.1. *Inhibition of TET proteins in hypoxia*

A hypoxic microenvironment can usually be diagnosed in most solid tumours [142,143]. The high metabolic activity of cancer cells can cause local oxygen and nutrient deficits (tumours larger than 2 mm in diameter). Oxygen deficiency led to the activation of HIF1s such as HIF-1α via lower activity of oxygen-dependent proline hydroxylase (repressor of HIF-1α). HIF-1α stimulates the expression of numerous oncogenic genes, which control cell survival, migration, and tumour development. Hypoxia shows a strong correlation between metastatic activity and loss sensitivity to treatment, therefore its targeted control is highly desirable. Some high-impact research works strongly suggest that TET proteins could play an important role in the hypoxia phenotype (Fig. 6) [144]. Targeting the TET protein could represent a promising strategy in the treatment of hypoxic tumours.

Although an increase in DNA methylation is usually a phenomenon in various tumours, the hypoxia phenotype causes a decrease in 5mC in cancer cells [145,146]. In contrast, in human cardiac fibroblasts, hypoxia leads to higher DNMT1 and DNMT3B and global DNA hypermethylation [147]. In breast cancer cells, HIF-1α can stimulate the expression of the TET1 and TET3 proteins [148]. Down-regulation of either TET1 or TET3 reduces the CD44 + /CD24 subpopulation and EMT in cells overexpressing HIF-1α. Silencing of TET1 and TET3 proteins causes their increased sensitivity to paclitaxel. In a mouse model, tumours with silenced Tet1 and Tet3 proteins show slower growth and metastatic activity. Similarly, in breast cancer patients, TET1, TET3, and 5hmC levels correlate with tumour hypoxia, tumour aggressiveness, and poor prognosis. In hypoxic neuroblastoma cells, TET1 protein depletion decreases the expression of PGKI, HK2, CA9, BNIP3, and VEGFA [149]. Conversely, CpG demethylation in the HIF-1α promoter increases its

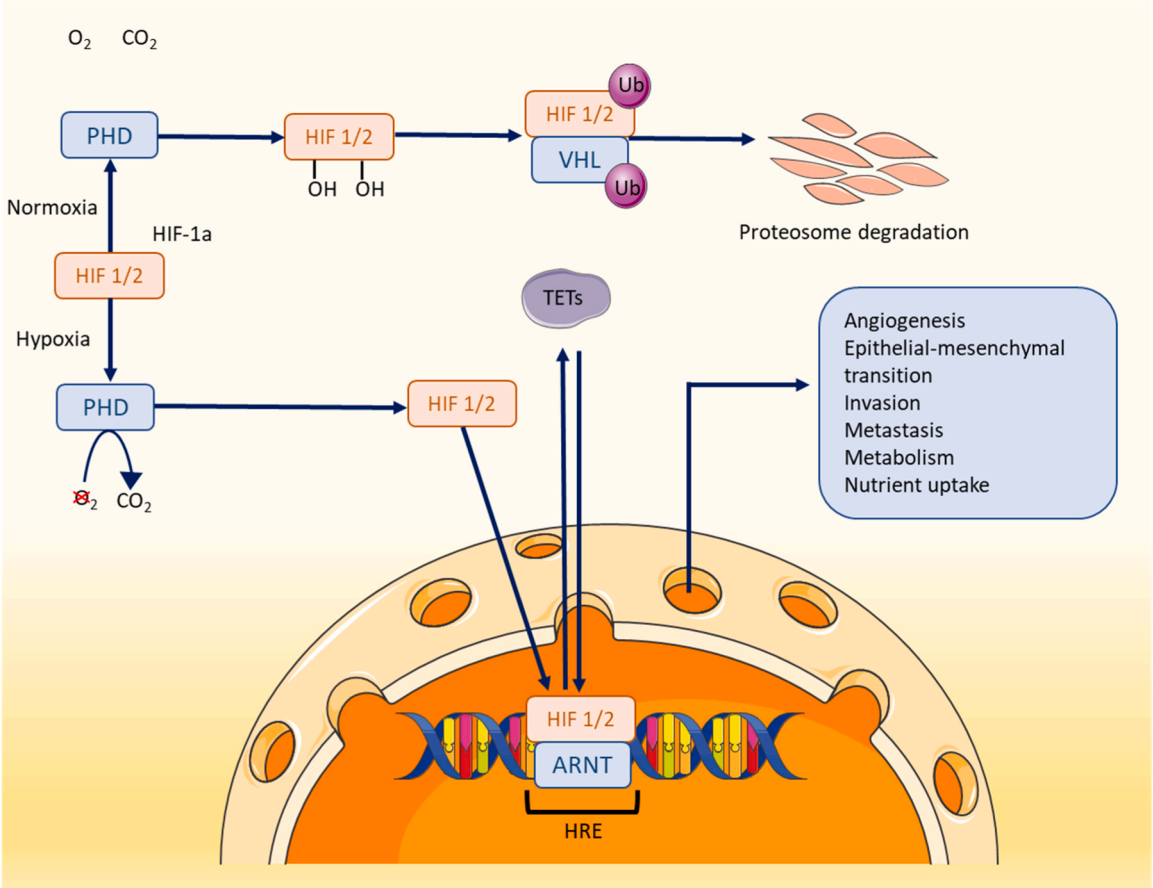

Fig. 6. Role of TET proteins in hypoxia. In normoxia, HIF 1/2 are hydroxylated by PHD and subsequently degraded with the assistance of Ub and VHL (a component of an E3 ubiquitin ligase complex). In hypoxia, lower oxygen levels did not enable HIF 1/2 hydroxylation and degradation. Non-degraded HIF 1/2 uptake into the nucleus and stimulate expression of hypoxia-induced genes, including TET1 and TET3. On the other hand, TET proteins such as TET proteins can stimulate the activity of hypoxia-induced genes, although this effect cannot be associated with the enzymatic activity of TET proteins. **Abbreviations**: HIF 1/2, *Hypoxia-inducible factor 1/2; PHD, Prolyl Hydroxylase; Ub, ubiquitin; VHL,* von Hippel-Lindau tumour-suppressor protein. The figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

expression and activity [150]. However, it should also be mentioned that the TET1 protein, specifically its CXXC domain, can directly interact with HIF-1α and HIF-2α [151]. In H1299 cells, knockdown of the TET1 protein suppresses hypoxia-induced EMT, but the expression of a catalytically inactive mutant of the TET1 protein restores its activity.

The above could indicate the limited possibility of TET protein inhibitors in targeting the hypoxic phenotype of cancer cells. On the other hand, hypoxia is a complex phenotype that can modulate the activity of other oncogenic factors such as NF-κB and vice versa [152]. A definite conclusion cannot be made without further biological studies, especially in vivo.

6.2. Inhibition of TET proteins in the cancer-immune system

TET proteins, especially the TET2 protein, play an important role in controlling the immune system [153–155]. TET2 protein expression and activity is strongly associated with the repression of inflammatory genes. For example, Zhang et al. reported that loss of TET2 protein strongly reduces IL-6 meditated transcription during the resolution of inflammation in innate myeloid cells, including dendritic cells and macrophages [156]. Higher activity of inflammatory factors such as IL-6 was observed in Tet2-deficient mice exposed to dextran sulfate sodium [156].

Targeting TET protein functionality could be used to modulate the immune system in oncogenic patients. Lie et al. reported that CpG demethylation in Foxp3 promotor, most probably induce by TET proteins, may be significantly involved in the stabilization of Foxp3 expression in Treg cells [157]. Along these lines, Chen et al. reported that a combination of sodium butyrate (an HDAC inhibitor), UC0646 (a histone methyltransferase inhibitor), and ascorbic acid (inductor of TET protein activity) induces complete demethylation of CNS2 region at the Foxp3 locus [158] Activation of Foxp3 signalling led to the conversion of CD4 + T effector cells to Foxp3 + Treg cells with a strong immunosuppressive phenotype. Treg cells enhance immune surveillance of tumours and suppress antitumour immune responses [159]. However, CD4 + T cells are more associated with anti-cancer immunity [160].

TET1 mRNA and TET1 protein levels were significantly reduced in activated macrophages (*P. gingivalis* LPS/IFN-γ and *E. coli* LPS/IFN-γ) [161]. However, *TET1 siRNA* suppresses NF-KB signalling pathways, M1 macrophage polarization, and inflammatory cytokine production. Similarly, inhibition of TET proteins (mostly TET1) by Bobcat339 strongly suppresses IL-1β–induced production of IL-6 and IL-8 in MCF-7 cells [123]. IL-1β is well known to activate NF-KB signalling, which stimulates cytokine production [162]. Consistent with the above, pre-treatment of HeLa cells with DMOG (1 mM) strongly reduced IL-1β–induced NF-KB activity in a time- and dose-dependent manner [163]. Although it should also be considered that DMOG inhibits multiple α-ketoglutarate-dependent dioxygenases, not just TET proteins.

Since the immunosuppressive effect of the TET2 protein is stimulated by ascorbic acid, its inhibitors could represent promising agents for the reactivation of immune cells in the tumour microenvironment. The above research studies also show that the activation of NF-κB signalling is associated with the enzymatic activity of the TET1 protein. p65 (a member of the NF-KB family) was found to be a negative regulator of the TET1 promoter in breast, lung, and melanoma cancer cells [164]. This could suggest that targeting its residual activity could sensitize tumours to treatment and reduce their metastatic potential. NF-KB is a key regulator of inflammation and immune responses [165,166]. In carcinogenesis, it stimulates treatment resistance, invasion, angiogenesis, and metastatic activity. The possible therapeutic application of TET protein inhibitors may require their significant selectivity not only for the targeted TET protein but even for the cellular phenotype. For example, the M1 macrophage phenotype exhibits strong antitumour activity, and its repression could be counterproductive in anticancer treatment.

7. Future direction

TET protein inhibitors are promising therapeutic agents. Currently, they are mostly studied for the treatment of oncological diseases [119]. In the future, these agents can be expected to be tested for the treatment of metabolic diseases such as diabetes mellitus or chronic schizophrenia. On the other hand, the possible inhibitory activity of used drugs and bioactive compounds (e.g., Eltrombopag [104] and cannabinoids [107]) against TET proteins must be taken into account to evaluate their therapeutic and side effects. These topics are discussed in more detail below, including possible limitations (e.g., specificity of TET protein inhibitors and intracellular localization of TET proteins).

Some high-impact publications suggest the possible potential of TET protein inhibitors in the therapy of non-oncological diseases. It is well known that 5hmC dysregulation is an important part of the pathogenesis of diseases such as neurological disorders (schizophrenia, Alzheimer's disease, and others) [167–169]. For example, the postmortem brains of chronic schizophrenia show significant increases in DNMT1, TET1 protein, 5mC, and 5hmC [167]. In the treatment of neurodegenerative diseases such as chronic schizophrenia, valproic acid is standardly used for a long time. However, its long-term application may cause significant side effects [170]. Palsamy et al. [171] reported that endoplasmic reticulum stress (induced by valproic acid) can cause passive and active demethylation in human lens epithelial cells. The levels of DNMT1, DNMT3a, and DNMT3b proteins are decreased (unlike mRNA) and the TET1 protein is increased [171]. In Neuro2a cells, valproic acid increased TET1 and TET2 protein levels in the nucleus, and neuronal differentiation was inhibited [172]. Although 5hmC was increased, the observed effect was also seen in an enzymatically inactive mutant of the TET1 protein. Overexpression of TET2 protein and an increase in 5hmC were also observed in phase-arrested and proliferative HeLa cells treated with valproic acid [173]. On the contrary, in the case of the mitochondrial epigenome, valproic acid reduces the mitochondrial level of Tet1 protein and 5hmC in mouse 3T3-L1 cells [75]. On the other hand, it cannot be ruled out that the increase in TET1 protein activity may be part of a compensatory mechanism. In a rat model of spinal cord injury, an increase in Tet2 protein and global 5hmC was observed in traumatic spinal cord tissues [174]. The application of SC-1 (an inhibitor of TET2 expression) significantly increased the necrotic volume.

Although the enzymatic activity of TET proteins is involved in the mechanism of the disorder, it may not always be the most appropriate target for treatment. Interferon-inducible 44 like (IFI44L) is one of the interferon-stimulated genes upregulated in many autoimmune diseases (such as systemic lupus erythematosus (SLE)) [175]. Monocytes obtained from patients with SLE showed significantly higher expression of STAT3 [176]. STAT3 recruits the protein TET2, which demethylates the IFI44L promotor. Its overexpression induces the differentiation of monocytes into dendritic cells (one of the key players in the pathology of SLE). The *TET2 mRNA* level in monocytes derived from patient cells was not different from the healthy control [177].

Li et al. reported that the TET2 protein could be significantly involved in atherosclerosis [178]. Atherosclerosis (non-resolving inflammatory disease) is a well-known common cause of myocardial infarction and stroke [179]. Macrophages and oxidized low-density lipoprotein (OxLDL) accumulate in the subendothelial space. Macrophage apoptosis promotes plaque necrosis, which promotes acute atherothrombotic cardiovascular events [180,181]. Apoptosis of macrophages can be suppressed by autophagy. However, THP-1 macrophages treated with OxyLDL show higher expression of the TET2 protein, which suppresses the induction of autophagy [178]. Application of TET2 siRNA or 5-Aza-2′-deoxycytidine suppresses the effect of OxLDL on autophagy [178].

Dysregulation of the TET proteins activity is also associated with the pathogenesis of diabetes mellitus. Significantly increased amounts of TET2, matrix metalloproteases 9 (MMP-9), and α-KG, whose levels are related to local hypoxia and poor glycaemic control, were found by Tan

et al. [182] in diabetic wounds compared with nondiabetic wounds. It has been observed that elevated MMP-9 levels can be associated with suppression of wound healing, mainly in diabetes mellitus [182–184]. Genomic DNA methylation profiling of type 2 DM islets revealed that CpG loci exhibited a substantial hypomethylation phenotype such as retinal MMP-9 promoter, which may provide insight into DNA methylation and diabetic pathogenesis (e.g., foot ulcers and diabetic retinopathy) [182,185–187]. TET2 displays significant affinity to a fragment surrounding the transcriptional start site in the MMP-9 promoter region [188]. In addition, evidence indicated that TET2 affected the migration and proliferation in vitro of cultured skin primary keratinocytes [188].

Impaired healing of diabetic wounds could be also associated with TET1. DNA demethylation regulatory protein GADD45a (strongly upregulated in diabetes) binds to the promoter of MMP-9 and recruits thymine-DNA glycosylase for base excision repair-mediated demethylation in diabetic HaCaT cells and diabetic rat skin [185]. Nevertheless, GADD45a can also directly interact with TET1 protein and stimulate 5mC oxidation [189].

The above suggests that in some cases inhibitors of TET proteins could show promising therapeutic potential. Their application could offer a more selective modulation of TET proteins than their down-expression. However, target disruption may be associated with a different functionality of TET proteins, which may be severely limited by enzyme activity and the utility of their inhibitors.

On the other hand, it can be assumed that several clinically applicable substances can also show inhibitory effects against TET proteins. For example, the clinically used drug Eltrombopag is also a potent inhibitor of the TET proteins [104]. It cannot be excluded that this inhibitory activity can also be observed with other drugs used. Other iron chelators can be mentioned in this context, but the question is whether their effect is related to the chelating ion Fe(II) rather than enzyme targeting.

From the results of Singh's study, it can be concluded that the biological effect of natural phenolic compounds could also be associated with the inhibition of TET proteins [108]. Many of them are known to be strong Fe(II) chelators and the biological effect of their metals, especially iron complexes, is much debated topic. It is possible that some natural phenolic compounds or their Fe(II) complexes could target TET proteins. For example, we found that cannabidiol and cannabinol showed strong affinity for Fe(II) ions and strong inhibitory activities (IC_{50} = 4.8 and 6.27 μM, respectively) against the TET1 protein [107]. In this context, it should be mentioned that co-applications of valproic acid and CBD are beginning to be studied in clinical trials [190–192]. However, CBD restores mtDNA hydroxymethylation in iron-treated rats [85]. It may be a strong limitation of the presented hypothesis, however, valproic acid against the mitochondrial epigenome also acted against the nuclear one [75].

The discussed results also suggest that the anticancer effect of some polyphenols, especially flavonoids, could be associated with the inhibition of TET proteins. However, in a melanoma cell and mouse model, the effect of quercetin was dependent on TET1 protein expression [193]. However, therapeutically effective targeting of TET proteins will undoubtedly require an enormous effort to develop new specific inhibitors [92,109,110].

However, the above assumed that 5mC oxidation is the exclusive mechanism for the formation of 5hmC in DNA. Chen et al. reported that DNMT3A and DNMT3B and the slower DNMT1 also exhibit dehydroxymethylase activity [10]. In this case, the mitochondrial epigenome is one of the discussed mechanisms of mtDNA demethylation [194]. DNMT1 is the only DNMT with a mitochondria-targeting sequence [71]. However, cytoplasmic Dnmt3a showed considerable localization to mitochondria in NSC34 cells [195]. DNMT3B was also observed in the mitochondrial fraction of human and mouse cells. In addition, Liutkeviciute et al. reported that S-adenosylmethionine-dependent DNA methyltransferases (DNMT1) using exogenous aldehyde can produce the corresponding 5-(α-hydroxyalkyl)cytosine via exogenous aldehyde, which incorporates 5hmC into DNA [196]. In mouse zygotes, depletion of the TET3 protein did not decrease 5hmC levels. However, 5-aza-2'--deoxycytidine (Decitabine; DNMT inhibitor) causes a significant decrease in 5hmC levels in the paternal pronucleus [55]. The DMOG application caused a decrease in 5hmC but did not affect the level of 5mC. However, the treatment with 5-aza-2'-deoxycytidine can lead to the demethylation of the TET2 promotor and an increase in its expression [129,173].

Briefly, the above findings might suggest a strong therapeutic potential of TET protein inhibitors. Nevertheless, certain limitations should be considered. First, TET proteins also exhibit a non-enzymatic effect on gene expression. In this case, their inhibitors are unlikely to be used. Second, some studies discussed above suggest that the enzymatic activity of the TET protein is not exclusive to 5mC, 5hmC, and 5fC in DNA. The effect of their inhibition could be much broader than expected. Third, the role of other enzymes in DNA demethylation cannot be ruled out. Their inhibition cannot lead to the expected effect. Fourth, effective therapy may require targeting only one type of TET protein or better one of its isoforms. Currently, an inhibitor with specificity for TET1 and TET2 proteins, but not for TET3 proteins, has been prepared. And more importantly, only the recombinant catalytic domains of TET proteins were used to determine enzymatic activity. Their effect against individual isoforms cannot be sufficiently predicted. Fifth, our knowledge of their role in the regulation of gene expression, in pathogenesis, and especially in mitochondria is limited. We may not have enough information for design therapies. Further intensive research is necessary for the therapeutic application of TET protein inhibitors.

8. Conclusion

Numerous studies have shown a significant correlation between the dysregulation of TET proteins and the pathogenesis of many serious diseases. TET protein inhibitors could be promising agents for regulating their activity. This review discusses their various mechanisms of inhibition and shows their usefulness and limitations, especially in the treatment of oncological diseases. The cited high-impact studies strongly suggest the high potential of this approach. However, our knowledge of the epigenetic mechanism is limited, especially in the case of mitochondria. Further biological and clinical studies are definitely needed to better understand the role of TET proteins and to design new therapeutic strategies based on their inhibitors.

Author statement

Robert Kaplánek participated in writing and supervision of manuscript, he also integrated individual author contributions. **Zdeněk Kejík** participated in writing and supervision of manuscript, design of figures and table preparations. **Jan Hajduch** participated in writing and supervision of manuscript. **Kateřina Veselá** and **Kateřina Kučnirová** participated in writing manuscript and table preparation. **Markéta Skaličková** and **Anna Venhauerová** participated in design of figures and preparing tables. **Božena Hosnedlová** participated in writing and supervision of manuscript. **Róbert Hromádka**, **Petr Dytrich** and **Petr Novotný** participated in writing and supervision of manuscript. **Nikita Abramenko**, **Veronika Antonyová**, **David Hoskovec**, **Petr Babula**, and **Michal Masařík** participated in writing and supervision of manuscript. **Pavel Martásek** and **Milan Jakubek** design concept of manuscript and participated in manuscript supervision.

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Declaration of Competing Interest

Robert Kaplánek has no conflict of interest. **Zdeněk Kejík** has no conflict of interest. **Jan Hajduch** has no conflict of interest. Kateřina Veselá and Kateřina Kučnirová has no conflict of interest. **Markéta Skaličková** and **Anna Venhauerová** has no conflict of interest. **Božena Hosnedlová** has no conflict of interest. **Róbert Hromádka** has no conflict of interest. **Petr Dytrych** has no conflict of interest. **Petr Novotný** has no conflict of interest. **Nikita Abramenko** has no conflict of interest. Veronika Antonyová has no conflict of interest. **David Hoshovec** have no conflict of interest. **Petr Babula** has no conflict of interest. **Michal Masařík** has no conflict of interest. **Pavel Martásek** has no conflict of interest. **Milan Jakubek** has no conflict of interest.

Data Availability

No data was used for the research described in the article.

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