Inhibitors of Glutamine Synthetase and their Potential Application in Medicine

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Abstract: Glutamine synthetase (E.C. 6.3.1.2) – an enzyme catalyzing formation of glutamine from glutamate and ammonium ion, is one of the most important enzymes in nitrogen metabolism. Due to glutamine synthetase activity, inorganic nitrogen is incorporated in the cell metabolism and is further used in biosynthesis of several highly important metabolites.

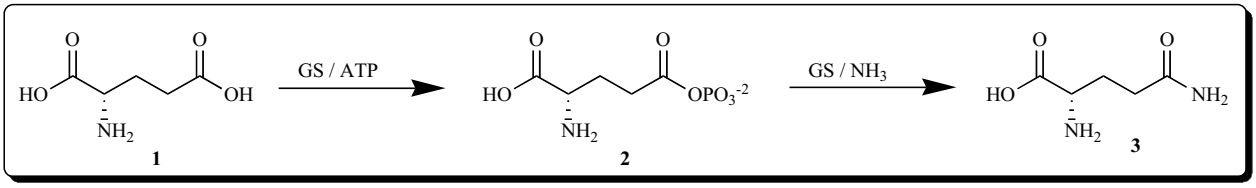
The first part of the review presents the long-dating research on inhibitors of glutamine synthetase which started with the discovery of methionine sulfoximine in 1949. Since that time several inhibitors of this enzyme, classified in the following groups: derivatives of methionine sulfoximine, phosphorus containing analogues of glutamic acid, bisphosphonates and miscellaneous inhibitors, have been developed and described. Analysis of their structure-activity relationships is presented in some detail.

The second part of the paper is dedicated to potential medical application of glutamine synthetase inhibitors, which proved to act as effective anti-tuberculosis agents with high selectivity towards the pathogenic bacteria. Moreover, it was also shown that glutamine synthetase inhibitors could be successfully applied in cancer therapy.

1. INTRODUCTION

Glutamine synthetase (GS, E.C. 6.3.1.2) is one of the most significant enzymes in nitrogen metabolism. It catalyses the conversion of glutamate (**1**) and ammonium ion to glutamine (**3**) in the presence of ATP [1].

Mechanism of the enzymatic reaction consists of two steps. Glutamate (**1**) is phosphorylated by ATP forming active intermediate — γ-glutamyl phosphate (**2**), which subsequently reacts with ammonia yielding glutamine (**3**). Glutamine amide nitrogen atom is then transferred by glutamate synthetase (GOGAT) to α-ketoglutarate forming two glutamate molecules. Thus, GS-GOGAT cycle allows incorporation of inorganic nitrogen in the cell metabolism.



GS is a large polymeric enzyme consisting of twelve subunits in case of bacterial proteins and ten in eukaryotic ones [2, 3]. There are twelve or ten active sites (respectively) located between two subunits. They are arranged in a form of bifunels with two entrances from opposite sides for binding glutamate and ammonium ion or ATP. Interestingly, the glutamate binding site is highly conserved among enzymes from different species, while that for ATP differs in proteins of bacterial and eukaryotic origin. Glutamate is bound to the enzyme with several hydrogen bonds and ionic interactions between its γ-carboxylate and metal ion (manganese(II) or magnesium(II)) present in the active site [4]. ATP molecule attaches from the opposite direction where its triphosphate unit interacts with all metal ions. Although most of the known GS inhibitors are analogues of glutamate, bisphosphonates constitue the only known example of synthetic inhibitors which interacts most probably with the ATP binding site.

In this review the development of GS inhibitors are discussed with particular attention paid to structure-activity relationships. Most of the known GS inhibitors are organosulfur or organophosphorus analogues of glutamate with methionine sulfoximine and phosphinothricin being the most remarkable examples. Moreover, several miscellaneous compounds mainly of natural origin that inhibit GS activity are also presented. The second part of the paper is dedicated to possible medical applications of GS inhibitors to tuberculosis and cancer treatment.

2. GS INHIBITORS

Great interest in GS research has led to discovery of several GS inhibitors. They can be divided into four groups:

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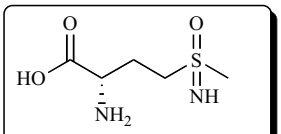
analogues of methionine sulfoximine, analogues of phosphinothricin, bisphosphonates, and miscellaneous inhibitors.

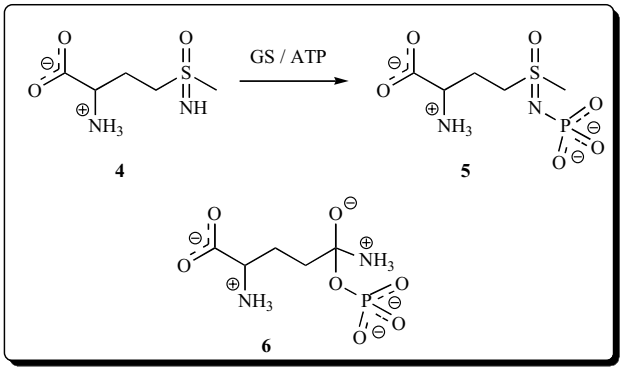
2.1. Analogues of Methionine Sulfoximine

Methionine sulfoximine (**4**) is the first GS inhibitor described [5]. The original reports concerning its biological activity were published in 1949 [6, 7], the structure was elucidated in 1950 [8, 9], while in 1952 it was proved that this compound is strong inhibitor of GS [5, 10-13].



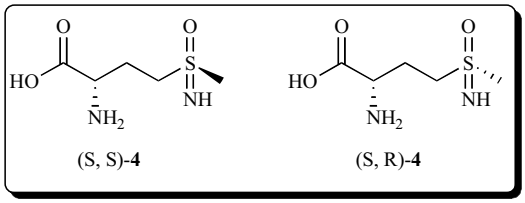
4, K_i = 2.0μM (*E.coli*) [10],
K_i = 210μM (sheep brain) [11],
K_i = 161μM (pea leaf) [12],
K_i = 100μM (spinach) [13].

The mechanism of GS inhibition by (**4**) was intensively studied, and it was shown that its irreversible mode of action results from phosphorylation inside the enzyme active site [14, 15]. The inhibitor phosphorylation process proceeds in the same way as glutamate phosphorylation during the native reaction with the use of ATP. Phosphorylated methionine sulfoximine (**5**) is the actual enzyme inhibitor, and only in this form it is bound practically irreversibly to the enzyme. This mechanism was proved additionally by application of methionine sulfoximine phosphate obtained synthetically [16]. The comparison of this structure to the enzymatic reaction transition state (**6**) allowed classifying it among the transition state analogues.



4 **5** **6**

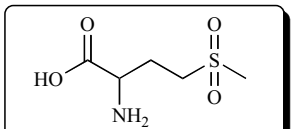
As expected, four stereoisomers of methionine sulfoximine exhibit significantly different inhibitory activities. Early studies of two mixtures of diasteroisomers: (S)-methionine (RS)-sulfoximine and (R)-methionine (RS)-sulfoximine indicated that only the first mixture inhibited the enzyme [15]. Subsequently, diastereoisomers of (S)-methionine (RS)-sulfoximine were separated chromatographically as salts with (+)-camphor-10-sulfonic acid and absolute configuration was determined using X-ray analysis [17]. It was shown that (S)-methionine (S)-sulfoximine was exclusively responsible for the enzyme deactivation [18]. Moreover, only one diastereoisomer of methionine sulfoximine phosphate (**5**) is produced upon treatment the enzyme with inhibitor diastereoisomeric mixture. Detailed research has showed that affinity of (S, S)-**4** to GS is 10 times higher that (S,R)-**4** [19]. Calorimetric studies on their binding demonstrated that this process was enthalpy driven and ΔH was similar for both diastereoisomers in case of unadenylylated *E.coli* enzyme, while it differed significantly for fully adenylylated GS [20].



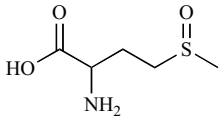
(S, S)-**4** (S, R)-**4**

The mode of binding of methionine sulfoximine to GS was examined by several techniques including calorimetry, EPR, NMR [20-22], but the final picture was given by the crystal structure of phosphorylated inhibitor (**5**) – enzyme (from *Mycobacterium tuberculosis*) complex [23]. This structure illustrates that inhibitor is bound very tightly with several hydrogen bonds and ionic interactions with metal ions present in the active site. Moreover, amino acid part of the inhibitor interacts very similarly to glutamate. The crystal structures of analogous complexes were recently obtained for maize and human enzymes [3, 24].

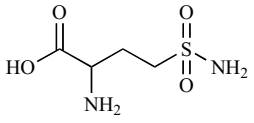
Several analogues and derivatives of methionine (compounds **7**-**15**) were obtained and their inhibitory activity against GS was evaluated [15, 25-27]. Despite high structural similarity of methionine sulfone (**7**) with methionine sulfoximine (**4**) they exhibited totally different mode of action, as the former compound appeared to be weak, reversible GS inhibitor [15, 25, 26]. Methionine sulfoxide (**8**) showed similar inhibitory kinetics as (**7**) but with lower affinity to the enzyme. Sulfonamide analogue of the lead (**4**) compound demonstrated high potency, but still lower than (**4**) [27].



7, K_i = 5mM (*E.coli*) [15, 26]

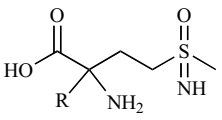


8, K_i > 5mM (*E.coli*) [15, 26]

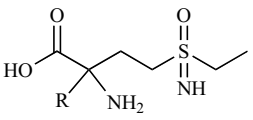


9, K_i = 51μM (*E.coli*) [27]

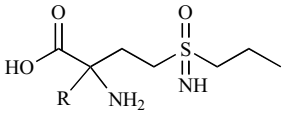
Several alkyl derivatives of methionine sulfoximine (**10**-**15**) were also obtained, but this approach to structure modification was unsuccessful. Inhibitory potencies of these compounds were generally low, and decreased with structural distance to the lead [28, 29].



10, R = CH_3,
$I_{0.1mM}$ = 80% [28],
11, R = C_2H_5,
$I_{0.4mM}$ = 41% [28].

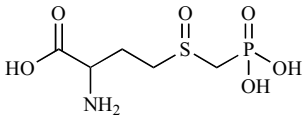


L-**12**, R = H,
I_{10mM} = 76.4% [29],
13, R = CH_3,
I_{2mM} = 25,8% [29].



14, R = H,
I_{20mM} = 35.8% [29],
15, R = CH_3,
I_{20mM} = 34.6% [29].

The introduction of nonhydrolysable methylene bridge between sulfur and phosphorus atoms into methionine sulfoximine phosphate (**16**, **17**) did not lead to enhancement of their inhibitory activity [30, 31]. Similarly, phosphonate analogue of methionine sulfoximine (**18**) did not show interesting enzyme affinity [32].



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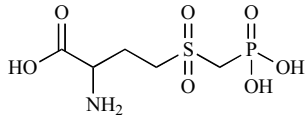
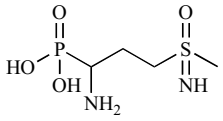


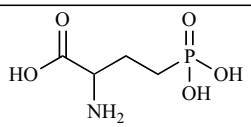
17, K_i = 1.4mM (sheep brain) [30],
$I_{0.5mM}$ = 51% (spinach) [31].



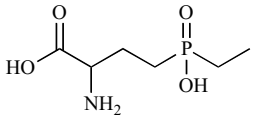
18, K_i = 1.2mM (*E.coli*) [32]

2.2. Phosphinothricin Analogues

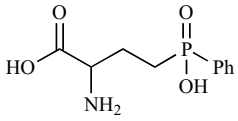
First organophosphorus inhibitors of GS were synthesized and evaluated by Mastalerz in 1959 (compounds **19**-**21**) [33, 34]. The idea was based on the similarity of the phosphonate/phosphinate group to the transition state of enzymatic reaction (**6**). It was found that *P*-ethyl γ-phosphinic analogue of glutamic acid (**20**) exhibited high inhibitory potency, while the phosphonic one (**19**) was only moderate GS inactivator. *P*-Phenyl analogue (**21**) appeared to be a very weak inhibitor of GS, due to steric impairment of the structure of the compound and the enzyme active site.



19, K_i = 54μM (*E.coli*) [27],
K_i = 3mM (pigeon liver) [33].

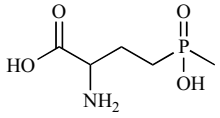


20, K_i = 50μM (pigeon liver) [33]

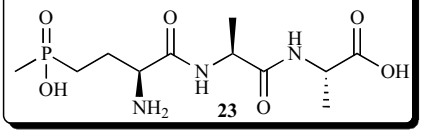


21, I_{40mM} = 30% (pigeon liver) [33]

The milestone in GS inhibition research was the isolation of phosphinothricin (**22**) from *Streptomyces viridochromogenes* [35, 36]. It is worth to note that that discovery was done simultaneously and independently by two research groups: the German and the Japanese one. It was found that these bacteria produced a tripeptide — L-phosphinothricyl-L-alanyl-L-alanine (**23**), which released strong GS inhibitor — compound (**22**) upon hydrolysis. It ranks among the most



22, K_i = 1.1μM (*E.coli*) [10],
K_i = 25.2μM (sheep brain) [37],
K_i = 8μM (sorghum seed) [38],
K_i* = 0.22nM (mung bean) (L-enantiomer) [46],
K_i = 3.0μM (spinach leaf) [39],
K_i = 320μM (rat liver) [40],
K_i = 73μM (pea leaf) [12],
K_i = 3.5μM (Barley GS1) [41],
K_i = 6.0μM (Barley GS2) [41],
K_i = 8.5μM (*Cassia* GS2) [41],
K_i = 2.0μM (maize GS1) [41],
K_i = 4.0μM (maize GS2) [41],
K_i = 8.0μM (soya GS2) [41],
K_i = 6.5μM (sugar beet GS2) [41],
K_i = 9.0μM (wheat GS2) [41],
K_i = 5.0μM (wild oats GS2) [41].



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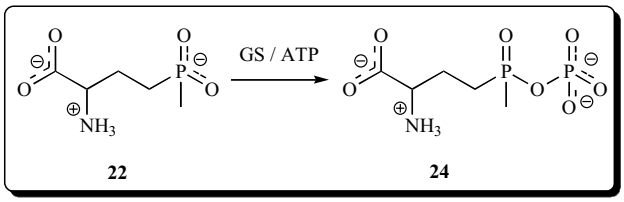
potent effectors of the enzyme, with K_i against *E.coli* enzyme estimated to 0.6μM for L-enantiomer [10, 12, 37-41]. It is worth to note that most of obtained K_i values are in low micromolar range and do not vary significantly (the value of K_i*=0.22nM differs from others due to definition of K_i*), what is the result of highly conservative structure of glutamate binding site (this phenomenon was confirmed by sequence alignment [1] and crystal structures [23, 24]).

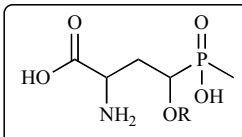
Detailed studies on the mode of GS inhibition by phosphinothricin have shown that it is similar to that found for methionine sulfoximine (**4**) [42]. Thus, the phosphinate oxygen atom of (**22**) is phosphorylated by ATP upon binding to the enzyme active site forming structure (**24**), which is the actual nearly irreversible inhibitor. The phosphorylation process was additionally proved by ^{31}P NMR analysis [43]. Recently published crystal structure of the complex of phosphorylated phosphinothricin (**24**) and GS revealed its highly similar mode of binding to methionine sulfoximine phosphate [3]. Interestingly, the phosphonate analogue (**19**) is also phosphorylated by ATP in GS active site, but contrary to phosphinothricin phosphate, it is released to the solution [10].



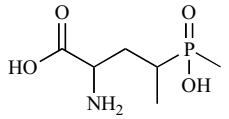
22 **24**

Since the discovery of phosphinothricin in 1972, several analogues of this compound have been obtained and evaluated for their GS inhibitory properties. Four main types of modifications could be distinguished: substitution in α or γ position, cyclization and phosphinic moiety modification.

The introduction of an additional γ-substituent to the phosphinothricin structure was proposed on the basis of results showing that GS was able to convert γ-substituted analogues of glutamic acid [44]. This assumption was experimentally verified for a series of compounds (**25-27)** showing their high affinity to the enzyme [10, 11, 38, 39, 45, 46]. γ-Hydroxyphosphinothricin (**25**) was the most active compound in this group with K_i = 1.6μM for enzyme from *E. coli*, what was comparable with phosphinothricin (K_i = 1.1 μM).

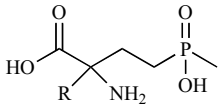


25, R = H,
K_i = 1.6μM (*E.coli*) [45],
K_i = 47μM (sheep brain) [11],
K_i = 14μM (sorghum seed) [38],
K_i* = 0.8nM (mung bean) [46],
K_i = 12μM (spinach leaf) [39],
26, R = $COCH_3$,
K_i = 33μM (*E.coli*) [45],
K_i = 178μM (sorghum seed) [38].

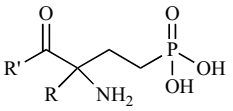


27, K_i = 25μM (*E.coli*) [10],
K_i = 347μM (sheep brain) [11],
K_i = 67μM (sorghum seed) [38],
K_i* = 140nM (mung bean) [46],
K_i = 492μM (spinach leaf) [39].

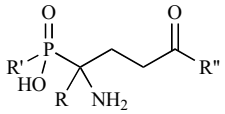
Another type of modification of the lead structure of phosphinothricin has been envisaged by introduction of α-substituent yielding compounds (**28)** and (**29)** [10, 37, 39, 40]. Both inhibitors exhibited high potency, however substantially lower than the parent compound. The appropriate phosphonate analogues were also prepared — structure (**30**) and its derivatives (**31**) and (**32**), but they showed poor activity [40]. Compounds (**33-38**) could be considered as analogues of α- or γ-substituted phosphinothricins, but their distant similarity to the lead structure was the reason of their low potency [40].



28, R = CH_3,
K_i = 5.7μM (*E.coli*) [10],
K_i = 125μM (sheep brain) [37],
K_i = 24μM (sorghum seed) [39],
K_i = 16μM (spinach leaf) [39],
K_i = 56μM (rat liver) [40],
29, R = C_2H_5,
K_i = 111μM (sheep brain) [37],
K_i = 22μM (sorghum seed) [39],
K_i = 32μM (spinach leaf) [39].

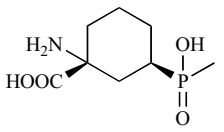


30, R = CH_3, R' = OH,
K_i = 6.3mM (rat liver) [40],
31, R = H, R' = NH_2,
K_i = 6.2mM (rat liver) [40],
32, R = H, R' = OCH_3,
K_i = 8.7mM (rat liver) [40].

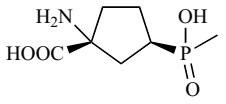


33, R = H, R' = OH, R" = OH,
K_i = 1.3mM (rat liver) [40],
34, R = H, R' = CH_3, R" = OH,
K_i = 0.79mM (rat liver) [40],
35, R = CH_3, R' = OH, R" = OH,
K_i = 16mM (rat liver) [40],
36, R = CH_3, R' = CH_3, R" = OH,
K_i = 9.5mM (rat liver) [40],
37, R = H, R' = OH, R" = NH_2,
K_i = 5.5mM (rat liver) [40],
38, R = H, R' = OH, R" = OCH_3,
K_i = 2.1mM (rat liver) [40].

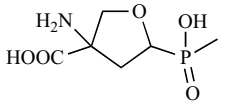
The introduction of an aliphatic cycle to the parent compound lead to novel GS inhibitors (**39-41**) [10, 37, 39, 46]. This modification caused reduction of conformational space of the inhibitor, thus entropic factor of Gibbs free energy of binding was smaller. The most active compound (**40**) showed K_i* = 0.47nM (against the mung bean enzyme), what was very close to the value found for phosphinothricin (K_i* = 0.22nM) under the same experimental conditions.



39 (racemic mixture),
K_i = 24μM (*E.coli*) [10],
K_i = 125μM (sheep brain) [37],
K_i = 20μM (sorghum seed) [39],
K_i = 72μM (spinach leaf) [39],
K_i* = 310nM (mung bean) [46].

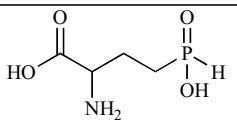


40 (racemic mixture),
K_i* = 0.47nM (mung bean) [46].

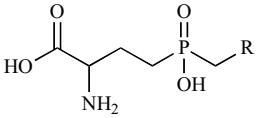


41, K_i* = 5.0nM (mung bean) [46].

Modification of methyl substituent of phosphinic group of phosphinothricin was also explored. The first attempt done by random screening (structures **42** – **48**) did not provide highly potent compounds [47, 48]. Only the closest structural analogue (**42**) of the parent compound bearing hydrogen atom replacing the methyl group, was found to be moderately active.



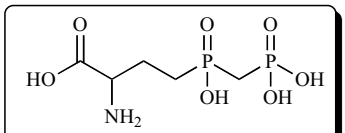
42, $I_{12.5mM}$ = 65% (pea leaf) [47]



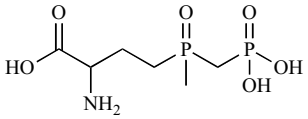
43, R = COOH,
$I_{12.5mM}$ = 35% (pea leaf) [47],
44, R = 3,4-dichlorophenyl,
$I_{12.5mM}$ = 20% (pea leaf) [47],
45, R = 3,5-dimethylphenyl,
$I_{12.5mM}$ = 20% (pea leaf) [47],
46, R = 4-bromophenyl,
$I_{12.5mM}$ = 20% (pea leaf) [47],
47, R = phenyl,
$I_{12.5mM}$ = 30% (pea leaf) [47],
48, R = $CH_2CH(NH_2)COOH$,
$I_{12.5mM}$ = 10% (pea leaf) [47].

The idea of modification of the phosphinic fragment of the phosphinothricin molecule by incorporation of an additional phosphonate group in order to construct a structure resembling phosphinothricin phosphate (**24**) yielded compounds (**49**) and (**50**) [49, 13]. Unfortunately, the obtained compounds were significantly less potent than the lead structure. This negative result could be the consequence of significant electronic difference between two phosphorus bridging entities — oxygen atom in case of structure (**24**) and methylene for (**49**) and (**50**), respectively.

Recently computer-aided studies on possibilities of modifications of the phosphinothricin methyl group revealed that introduction of positively charged substituent could represent novel, attractive approach to construction of GS inhibitors

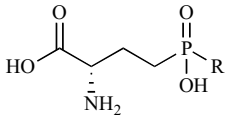


49, K_i = 75μM (*E.coli*) [49],
K_i = 750μM (sheep brain) [49],
K_i = 250μM (pea seed) [49].



50, K_i = 2.2mM (pea) [13].

[50]. It results from the fact that this methyl group docks near the negatively charged ammonium ion binding site. To verify this idea a series of compounds (**51**-**55**) was prepared. Evaluation of their inhibitory properties confirmed the working thesis ranking them among the most potent inhibitors developed so far [50, 51]. The highest inhibitory potency was discovered for the least extended analogue (**51**), with K_i = 0.59μM against *E. coli* enzyme.

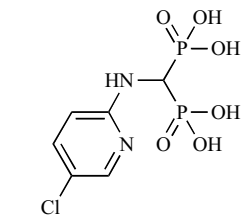


51, R = CH_2NH_2,
K_i = 0.59μM (*E.coli*) [50],
K_i = 7.9μM (spinach leaf, plastidial isoform) [51],
K_i = 1.8μM (spinach leaf, cytosolic isoform) [51],
52, R = CH_2OH,
K_i = 2.1μM (*E.coli*) [50],
K_i = 45.8μM (spinach leaf, plastidial isoform) [51],
K_i = 8.5μM (spinach leaf, cytosolic isoform) [51],
53, R = $CH_2(CH_3)NH_2$,
K_i = 3.7μM (*E.coli*) [50],
K_i = 17.6μM (spinach leaf, plastidial isoform) [51],
K_i = 21.6μM (spinach leaf, cytosolic isoform) [51],
54, R = $CH_2CH_2NH_2$,
K_i = 13μM (*E.coli*) [50],
K_i = 133μM (spinach leaf, plastidial isoform) [51],
K_i = 320μM (spinach leaf, cytosolic isoform) [51],
55, R = CH_2CH_2COOH,
K_i = 150μM (*E.coli*) [50].

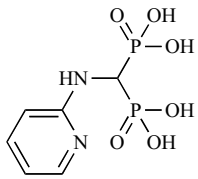
2.3. Bisphosphonates

Derivatives of aminomethylenebisphosphonic acid constitue another group of effective inhibitors of GS [52, 53]. Among studied compounds (**56**-**65**), analogue (**61**) appeared the most active exhibiting IC_{50} = 33μM against enzyme isolated from rice, what was comparable to the value obtained for D,L-phosphinothricin (IC_{50} = 44μM) [54].

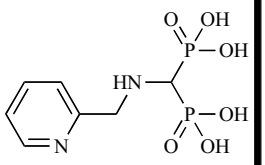
It is evident that this group of compounds is not structurally related to phosphinothricin and their mode of inhibitory action must be entirely different. Molecular modeling studies strongly suggest that they could be bound to the enzyme near



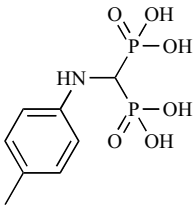
56, IC_{50} = 69μM (rice) [54]



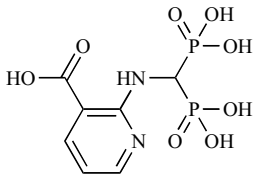
57, IC_{50} = 243μM (rice) [54]



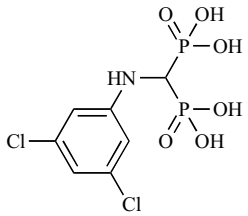
58, IC_{50} = 64μM (rice) [54]



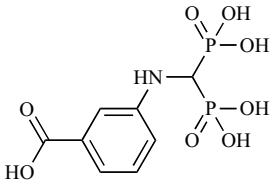
59, IC_{50} = 170μM (rice) [54]



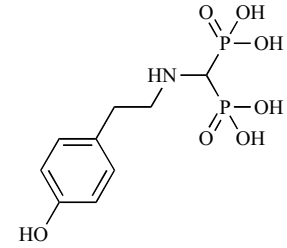
60, IC_{50} = 334μM (rice) [54]



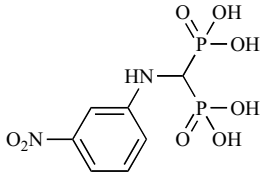
61, IC_{50} = 33μM (rice) [54]



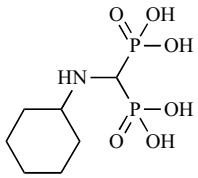
62, IC_{50} = 1.8mM (rice) [54]



63, IC_{50} = 4.47mM (rice) [54]



64, IC_{50} = 738μM (rice) [54]



65, IC_{50} = 1.1mM (rice) [54]

ATP binding site [55]. Bisphosphonic moiety interacts favorably with two metal ions present in the active site analogously to the triphosphate unit of ATP. As the consequence, this group of GS inhibitors is the only one, which is not structurally analogous to the glutamate substrate and does not interact with the glutamate binding site. It is noteworthy that contrary to the structure of glutamate binding site, ATP binding site differs in proteins from different species [24]. Thus, only compound that binds to this region could be specific to the enzyme of chosen origin.

2.4. Miscellaneous GS Inhibitors

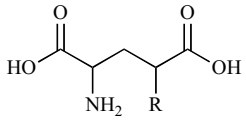
GS is a central enzyme of nitrogen metabolism and therefore its regulation is of vital importance [56]. There exist two regulatory mechanisms for this enzyme: adenylylation/deadenylylation process and inhibition by the end products of glutamine metabolism. Adenylylation/deadenylylation of tyrosine residue which causes significant changes of GS activity, is catalyzed by adenyltransferase. Control of adenyltransferase action by regulatory protein allows precise adjustment of GS activity. Additionally, several important metabolites are inhibitors of GS, namely: glycine (K_i = 0.8mM, *Salmonella typhimurium*), alanine (K_i = 0.16mM, *Salmonella typhimurium*), serine (K_i = 1.1mM, *Salmonella typhimurium*) [57], tryptophane, histidine, carbamoyl phosphate, glucosamine 6-phosphate, AMP (K_i = 0.85mM, *Salmonella typhimurium*) [58], CTP [59-61]. Early studies by Woolfolk and Stadtman suggested that each inhibitor bound to a distinct site, different from the enzyme active site. Accordingly, simultaneous action of mentioned compounds causes almost complete inactivation of GS, which was called "cumulative feedback inhibition". However, later studies with the use of the NMR technique showed that the amino acids are bound to glutamate binding site, while nucleotides to the ATP binding site [62]. Finally, the crystal structures of alanine, glycine and serine complexes with *Salmonella typhimurium* GS proved unambiguously that amino acid portion of these inhibitors interacted nearly identically with the analogous fragment of glutamate [57]. The crystal structure of AMP-GS complex also undoubtfully indicated harboring of AMP to ATP binding site [58].

Interestingly, the activity of GS in cyanobacteria is controlled in a different way [63]. Studies on the *Synechocystis* sp. enzyme showed that the regulation was governed by two

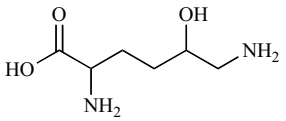
inhibitory peptides IF7 and IF17 [64]. Expression of *gif*A and *gif*B genes encoding these regulatory peptides depends on the concentration of ammonium ions in the cell. It is noteworthy that percentage of glutamine and arginine in these peptides is very high (13.7% Gln, 12.5% Arg for IF7). High concentration of nitrogen rich amino acids in the cell causes increased production of the regulatory peptide and subsequently inhibition of GS [65].

Binding of IF7 (K_D = 0.3±0.1μM) or IF17 to GS causes its complete deactivation. As the result of high content of positively charged amino acids, these peptides exhibit high isoelectric points: 11.2 for IF7 and 10.9 for IF17, respectively. pH dependent mode of IF7 and IF17 binding suggests that their interactions with the enzyme are mainly of electrostatic type. Moreover, it was proven that peptide IF7 was natively unfolded, what was crucial for its affinity to GS [66]. Reactivation of the enzyme relies on degradation of these inactivators, most probably by metalloproteases [67].

Several GS inhibitors structurally related to glutamate were either synthesized or isolated from biological sources. It was found that chemically obtained glutamate derivatives such as 4-hydroxyglutamate (**66**) and 4-fluoroglutamate (**67**) were weak inhibitors of GS from *Chlorella* [44].



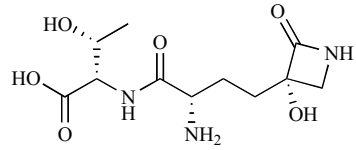
66, R = OH,
K_i = 1.42mM (*Chlorella*) [44],
67, R = F,
K_i = 0.62mM (*Chlorella*) [44].



68, K_i = 40μM (*S. typhimurium*) [1],
K_i = 490μM (maize GS1) [41],
K_i = 850μM (maize GS2) [41].

On the other hand, δ-hydroxylysine (**68**) — naturally occurring amino acid was also found to be a moderate GS inhibitor. First report concerning its biological activity was published in 1957 indicating regulation of glutamine and protein biosynthesis in carcinoma cells [68]. Later, it was evidenced that amino acid (**68**) inhibited the bacterial enzyme as well as both forms (GS1 and GS2) isolated from maize [1, 41].

Three interesting GS effectors were isolated from bacteria. Tabtoxine (**69**) is a dipeptide from *Pseudomonas syringae* pv. *tabaci*, bacterial species responsible for the Wildfire disease of tobacco plants [69, 70]. After hydrolysis of the peptide bond *in vivo* amino acid (**70**) is released. The kinetic data showed that compound (**70**) was effective, irreversible GS inhibitor, while the dipeptide (**69**) did not exhibit any activity and most likely served as transportation unit [71]. Further studies proved that ATP is indispensable for GS inhibition by (**70**) and its mode of action is very similar to that of methionine sulfoximine [72, 73].



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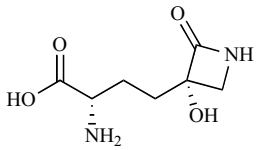
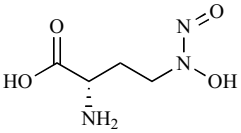
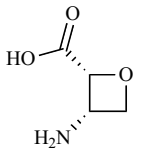


70, K_i = 1mM (pea seed) [73]

Two other weak inhibitors of GS possessing amino acid structures — alanosine (**71**) and oxetin (**72**) are produced by *Streptomyces* [74, 75]. Their inhibition constants lay in milimolar range.

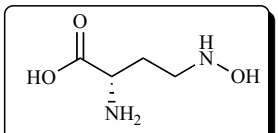


71, K_i = 1.3mM (mouse liver) [74],
K_i = 4mM (mouse brain) [74].



72, K_i = 1.2mM (*Bacillus subtilis*) [75],
K_i = 3.4mM (spinach leaf) [75].

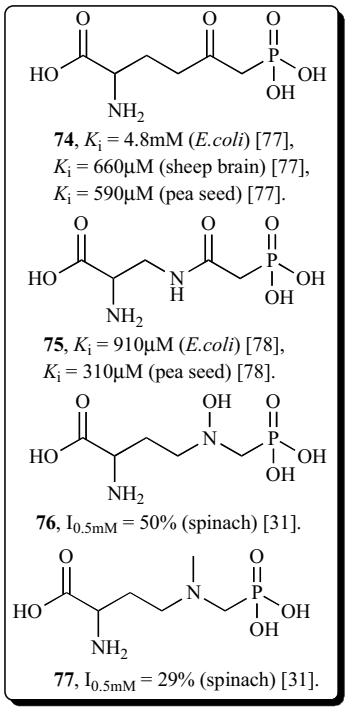
Analyzing the structures of methionine sulfoximine, phosphinothricin and δ-hydroxylysine, Nozoe and co-workers designed novel GS inhibitor — 2-amino-4-hydroxyaminobutyric acid (**73**) [76]. It was proved that it was potent inactivator of GSs with K_i being in micromolar range.



73, K_i = 21μM (soybean) [76],
K_i = 7μM (sheep brain) [76].

On the basis of the course of the enzymatic reaction, proceeding through intermediate γ-glutamyl phosphate, phosphonate compounds (**74**) and (**75**) were designed [77, 78], however they were found to be moderate GS inhibitors. Analogously to the described above phosphonomethyl de-

rivatives of methionine sulfoximine and phosphinothricin, structures (**76**) and (**77**) were obtained as those originating from inhibitor (**73**) [31]. Previous statement that phosphonomethylation of a lead structure did not yield more active compounds, was reproduced also in this case.



74, K_i = 4.8mM (*E.coli*) [77],
K_i = 660μM (sheep brain) [77],
K_i = 590μM (pea seed) [77].

75, K_i = 910μM (*E.coli*) [78],
K_i = 310μM (pea seed) [78].

76, $I_{0.5mM}$ = 50% (spinach) [31].

77, $I_{0.5mM}$ = 29% (spinach) [31].

3. POTENTIAL MEDICAL APPLICATIONS OF GS INHIBITORS

The crucial role of GS in nitrogen metabolism strongly suggests that its activity could be related to several diseases. As glutamate is an important transmitter in the central nervous system, GS activity is altered in neural diseases. Significantly elevated activity of this enzyme was found in brains of patients with schizophrenia [79-83], Alzheimer's disease [82, 84-86] as well as those with amyotrophic lateral sclerosis [87]. On the contrary, GS activity is decreased in case of Huntington's disease [88-90]. However, at the present stage of the research, it was not proven that inhibitors of GS could be useful drugs against mentioned diseases. This is most likely because they do not cross blood-brain barrier. Two more promising areas of possible medical application of GS inhibitors are connected with development of anti-tuberculosis and anti-cancer agents.

3.1. Anti-Tuberculosis Drugs

Tuberculosis is one of the most wide-spread infections with the highest mortality among diseases caused by a single pathogen [91]. Due to the multi-drug resistance strains of *Mycobacterium tuberculosis* — disease's causative agent, novel antituberculosis drugs are rapidly needed. Intensive efforts for finding new therapeutic strategies to treat tuberculosis have been undertaken. Structural genomics studies on *M. tuberculosis* are directed towards finding novel molecular targets for potential anti-tuberculosis drugs [92]. Among several possibilities found, GS is one the most promising target with known three-dimensional structure [23].

M. tuberculosis releases significant amounts of proteins to the extracellular space, among which GS is one of the most abundant. Thus, besides enzyme's well defined role in nitrogen metabolism, in case of pathogenic mycobacteria GS presence is crucial for biosynthesis of cell wall component — poly-L-glutamate/glutamine structure. This phenomenon is directly related to pathogenic properties of bacteria, thus the inhibition of GS appears to be very promising strategy against tuberculosis [93]. First, it was shown *in vitro* that GS inhibitor — methionine sulfoximine as well as antisense oligodeoxyribonucleotides specific to *M. tuberculosis* GS block biosynthesis of poly-glutamine/glutamate structures [94-96]. Additionally, it was proven that these agents inhibit growth of bacteria, showing GS importance in cell homeostasis. Importantly, methionine sulfoximine blocks selectively the growth of pathogenic *M. tuberculosis*, *M. bovis* and *M. avium*, while does not exhibit any affect on nonpathogenic microorganisms [94]. Thus, it was concluded that disruption of the bacterial cell wall integrity significantly amplified inhibitor uptake into cell. Subsequently, *in vivo* studies using demanding guinea pig model have proven that methionine sulfoximine was effective antibiotic *in vivo*. Moreover, it enhanced considerably anti-tuberculosis action of the known drug – isoniazid [97]. In spite of several advantages of this strategy, some drawback has to be considered. GS inhibitors are known epileptogenic agents, though this effect is strongly variable with animal species [98, 99]. While dogs are highly sensitive to methionine sulfoximine, monkeys and rats are not. In case of guinea pig model, the inhibitor toxicity was mainly caused by blocking γ-glutamylcysteine synthetase, what was reverted by application of ascorbate. Although, it is expected that humans are relatively insensitive to methionine sulfoximine, drug candidate should not be inhibitory towards γ-glutamylcysteine synthetase, should be poorly transported into the brain and exhibit high selectivity towards *M. tubeculosis* GS in comparison to the human enzyme.

3.2 Anti-Cancer Drugs

Nevertheless, the first report concerning the activity of GS in tumor cells was delivered by Rabinovitz and coworkers in 1957, anti-cancer application of GS inhibitors had been rarely studied [68]. Later it was proven that glutamine supply is essential for the growth of some cancer lines [100, 101]. In case of a number of glicoma-derived cell lines exogenous glutamine was limiting, while cell lines, grown in the absence of this amino acid, could be suppressed by GS inhibitors — γ-hydroxylysine or methionine sulfoximine. Subsequently, it was found that optimal strategy of human glicoma and medulloblastomas chemotherapy was the application of combination GS inhibitor and glutamine antagonist (6-diazo-5-oxo-L-norleucine or acivicin) [102]. The synergism of their actions allows blocking glutamine metabolism in cancer cells effectively. The significant advantage of this approach is very low concentration of methionine sulfoximine required, what is of high importance due to its dose-dependant central nervous system toxicity.

The second possible use of GS inhibitors in cancer chemotherapy is related to application of anti-tumor enzyme L-

asparaginase. The cytotoxic effect of this enzyme is based on asparagine starvation and it could be reversed by increased activity of asparagine synthetase — the enzyme which synthesizes asparagine from aspartate and glutamine. Thus, the adaptation of cancer cell to metabolic stress caused by L-asparaginase is based on enhanced activity of asparagine synthetase as well as GS, which is the enzyme that provides the substrate for asparagines biosynthesis [103]. It was showed that application of both L-asparaginase and GS inhibitor — methionine sulfoximine allowed efficient treatment of cancers resistant to L-asparaginase [104].

4. CONCLUSIONS AND PERSPECTIVES

The research concerning GS has long and vast history, what reflects its importance in the cell metabolism. GS inhibitors gained particular attention due to their potential applications, what yielded several published compounds. Although, their herbicidal activity was mostly investigated in the past years, nowadays new, extremely important areas of their medical application were indicated. Studies on both anti-tuberculosis and anti-cancer properties of GS inhibitors illustrate significance of their potential use. Importantly, recently published crystal structures of GS deriving form pathogens and human would allow rational development of novel, highly active and selective inhibitors.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

GS = Glutamine synthetase;

K_i = Inhibition constant;

K_i^* = $K_i^x(K_{react}/K_{inact})$, where K_{react} and K_{inact} are the enzyme reactivation and inactivation rate constants, respectively;

I_{10mM} = Percent of inhibition at given (e.g. 10mM) compound concentration;

IC_{50} = Inhibitor concentration that causes 50% decrease of enzyme activity;

ATP = Adenosine triphosphate;

GOGAT = Glutamate synthetase;

NMR = Nuclear magnetic resonance;

EPR = Electron paramagnetic resonance;

AMP = Adenosine monophosphate;

CTP = Cytidine triphosphate;

IF7, IF17 = inhibitory factor 7 or 17

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