Covalent inhibitors of GAPDH: from unspecific warheads to selective compounds

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ABSTRACT

Targeting glycolysis is an attractive approach for the treatment of a wide range of pathologies, such as various tumors and parasitic infections. Due to its pivotal role in the glycolysis, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) represents a rate-limiting enzyme in those cells that mostly, or exclusively rely on this pathway for energy production. In this context, GAPDH inhibition can be a valuable approach for the development of anticancer and antiparasitic drugs. In addition to its glycolytic role, GAPDH possesses several moonlight functions, whose deregulation is involved in some pathological conditions. Covalent modification on different amino acids of GAPDH, in particular on cysteine residues, can lead to a modulation of the enzyme activity. The selectivity towards specific cysteine residues is essential to achieve a specific phenotypic effect. In this work we report an extensive overview of the latest advances on the numerous compounds able to inhibit GAPDH through the covalent binding to cysteine residues, ranging from endogenous metabolites and xenobiotics, which may serve as pharmacological tools to actual drug-like compounds with promising therapeutic perspectives. Furthermore, we focused on the potentialities of the different warheads, shedding light on the possibility to exploit a combination of a finely tuned electrophilic group with a well-designed recognition moiety. These findings can provide useful information for the rational design of novel covalent inhibitor of GAPDH, with the final goal to expand the current treatment options.

1. INTRODUCTION

Covalent inhibitors are molecules that covalently bind their targets in a specific position leading to temporary or permanent inactivation. Historically, this covalent mode of action has long been considered alarming by the research teams of pharmaceutical industries, due to the mistaken impression that covalent inhibitors have an intrinsic propensity to uncontrolled reactivity. The principal concerns, including the risk of damages through either direct off-target interaction or haptenization of protein, with ensuing immune response, have been fuelled by several studies of organ toxicity carried out in the 1970s and 80s.[1] Remarkably, although a significant number of authorized drugs exploit covalent mode of actions, most of those were identified through phenotypic screening in biological assays, and their covalent mechanisms were discovered only afterwards.[2] Moreover, the growing preference of structure-based drug discovery approach over phenotypic screening led to a significant reduction of covalent inhibitors development in the turn of the millennium, due to the reluctance to voluntarily choose a covalent mechanism of action during the drug discovery processes.[3] Although the most popular covalent drugs were not rationally designed as covalent inhibitors, their well-established safety and efficacy profiles in hundreds of millions of patients over the years demonstrated that covalent acting compounds might represent a convenient possibility for drug discovery. In contrast to toxicophores, which are commonly bioactivated to a reactive metabolite that can bind with macromolecules such as proteins or DNA, causing a toxic effect, covalent drugs are compounds that typically exhibit their reactivity only when properly positioned in close proximity to the target nucleophile within a specific protein, thanks to the presence of a suitable recognition moiety. Thus, electrophilic moieties commonly found in reactive metabolites, such as quinones or epoxides, can be used for the design of targeted covalent inhibitors provided that they are coupled to a highly specific recognition moiety and that their reactivity is mitigated by proper chemical modification.[4-6] Needless to say, there are chances that covalent drugs may show off-target reactions, but the safety profile has to be assessed case to case as for any drug.[4] Despite the scepticism about the safety of covalent inhibitors, these drugs have made an impressive positive impact on human health.[7, 8] Notably, there is a growing interest in the development of innovative anticancer covalent drugs characterized by outstanding safety and efficacy compared to the old-generation toxic alkylating agents. Thus, in the past ten years a large number of covalent drugs have been approved by the FDA, including anti-cancers, antibiotics and antivirals.[9] The covalent mechanism of action of these drugs offers several intrinsic advantages. Unlike non-covalent inhibitors, for which there is an inescapable dependency of pharmacokinetic over pharmacodynamic, these two processes are entirely dissociated in covalent drug model. In particular, the duration of inhibitions, and consequently the effect, is dependent only on the protein re-synthesis rate, because the target residence time is long (for reversible covalent inhibitors) or even virtually infinite (for irreversible covalent inhibitors). Since the lifetime of the inhibited complex is directly proportional to its ligand efficiency, these inhibitors exhibit exceptionally high potencies[10], which can lead to lower and less frequent dosing. Hence, a quick metabolic clearance is preferred to minimize off-target interactions after the drug has inactivated the target protein.[4] Moreover, some covalent compounds can achieve the druggability of 'undruggable' enzymes by targeting shallow non-catalytic nucleophiles, which results in the selective target inhibition.[11]

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} E{\bullet}I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{-}I$$

Equation 1.

The general mechanism of action of a covalent inhibitor is shown in equation 1.[4] First, the inhibitor binds non-covalently to the target, then the warhead attaches to the reactive nucleophile residue of the protein forming a covalent complex (*E-I*). The reversibility, and therefore the lifetime of inhibited complex, is governed by k_{-2}. Reversible covalent inhibitors exhibit a $k_{-2} > 0$, while for the irreversible ones it is essentially zero. Thus, for the structure-based drug design of covalent inhibitors, both the non-covalent (K_i) and covalent (k_2) interaction with the target protein must be taken into consideration, aiming to their harmonious

combination. To wisely enhance the reaction rate, it is necessary to develop a compound with a recognition moiety able to selectively establish non-covalent interactions with the target in such a manner to position the reactive electrophilic warhead in the proximity of the targeted residue. In this way, it is possible to access promising inhibition rates avoiding the use of warheads with high intrinsic reactivity, which may undergo off-target interactions. Owing to these kinetic considerations, conventional IC_{50} measurements are not very useful for the assessment of potency of covalent inhibitors, since these values can change when measured at different incubation times.[12] For this reason, the time-consuming determination of k_{inact}/K_i (where $k_{inact} = k_2$) is preferred to perform rational SAR analysis.[13] Moreover, separate studies of the two constants can provide useful quantitative information to guide the SAR exploration.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key glycolytic homotetrameric enzyme that catalyzes the conversion of glyceraldehyde 3-phosphate (GAP) into 1,3-biphosphoglycerate (1,3-BPG), with the parallel reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH.[14] GAPDH amino acidic sequence is highly conserved across the phylogenetic scale.[15] Structurally, GAPDH is composed of three distinct features: the NAD^+ binding domain (Rossmann fold), the catalytic domain, and a feature known as "S-loop" that connects them.[16] In the active cavity, there are two distinct sites of interaction: the inorganic phosphate is accommodated in the so-called 'Pi' site, while the substrate GAP binds to the 'Ps' site, which is strictly defined and more specific in the space than the former.[17] Each subunit of human GAPDH contains three cysteine residues, of which only one has catalytic activity. That catalytic thiol has a lower pK_a and enhanced nucleophilicity due to the presence of a proximal histidine in the surrounding environment, acting as an acid-base catalyst. The catalytic cycle of GAPDH is depicted in detail in Fig. 1.

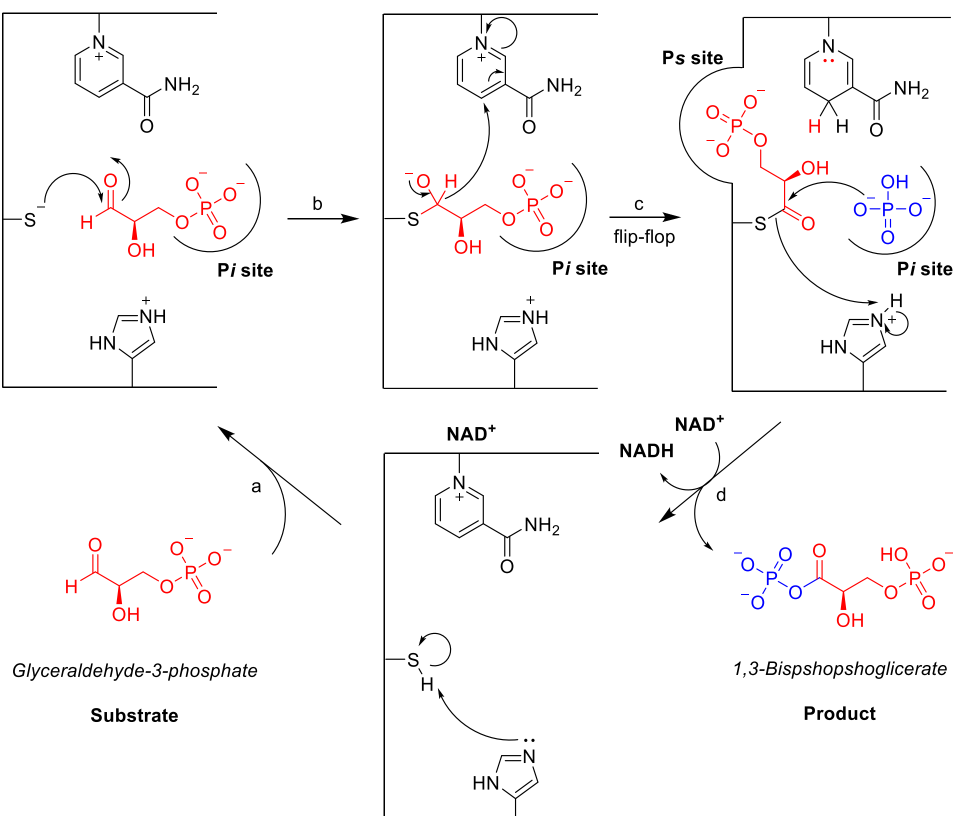


Figure 1. GAPDH enzymatic mechanism. The catalytic cycle consists of a multistep mechanism. First, the active form of GAPDH accommodates a molecule of GAP (a). Then the reactive cysteine residue performs a nucleophilic attack on the aldehyde group of GAP forming a thiohemiacetal intermediate (b). The reduction of NAD^+ to NADH leads to the conversion of the thiohemiacetal to thioester (c). During this step, the phosphate group is shifted to the P*s* site by a flip-flop mechanism.[17] This allows the inorganic phosphate ion to accommodate in the P*i* site and to attack the thioester, leading to the formation of the anhydride bond (acyl phosphate) and the consequent release of 1,3-BPG restoring the

deprotonated side-chain imidazole of the proximal histidine residue (d). Finally, the NADH cofactor in the enzyme is exchanged with its oxidized form NAD^+.

Due to its role in the catalytic mechanism, GAPDH activity can be abolished by covalent modification of the Cys residue that physiologically can be caused by endogenous compounds. For instance, the catalytic thiol is subjected to *S*-nitrosylation, followed by the binding of SIAH1 and nuclear translocation, which promotes apoptotic cell death.[18-21] Moreover, the thiol group can be modified by many endogenous substances, for example, it is sensitive to oxidation mediated by H_2O_2,[22] HOCl, taurine chloramine[23] leading to a loss of activity. Otherwise it can undergo reversible *S*-glutathionylation, which protects the catalytic thiol from the oxidative stress.[24] As a consequence, the catalytic cysteine of GAPDH represents an attractive target for the design of specific covalent inhibitors able to block the GAPDH catalyzed step of the glycolytic pathway. Since GAPDH possesses a pivotal role in the glycolytic pathway, it represents a rate-limiting enzyme in those cells that rely only or predominantly on glycolysis for energy production. In this context GAPDH inhibition can be a valuable approach for the development of anticancers,[25-29] immunomodulators,[30, 31] antibacterial[32] and antiparasitic drugs[33, 34]. The Warburg effect, which is known since 1924,[35] outlines the strict correlation between glycolysis and cancer and is considered the most prominent hallmark of cancer metabolism.[36, 37] Specifically, cells from most tumors, regardless of the tissue of origin, exhibit an exorbitant glucose consumption even in the presence of oxygen, resulting in elevated production of lactic acid. This contrasts with the behavior of healthy cells, which mainly generate energy from oxidative breakdown of pyruvate (TCA cycle and oxidative phosphorylation).[38-41] Remarkably, while hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) represent limiting steps of the glycolysis in physiological conditions, GAPDH becomes vital in the flux control of glycolysis only during the Warburg effect.[42] The inhibition of GAPDH is a strategic checkpoint for the complete breakup of the glycolytic pathway. Indeed, the previous steps catalyzed by HK and PFK proceed even when GAPDH is blocked by an inhibitor. Notably, PFK is activated when ATP level is lowered. Thus, ATP depletion induced by GAPDH inhibition causes additional ATP depletion due to positive feedback activation of PFK, which consumes ATP itself.[17] GAPDH is often overexpressed in cancer cells[43], becoming crucial for tumor survival, development, progression and propagation[44], both because of the Warburg effect, which allows tumor cells to grow and survive even during oxygen deprivation, as well as for several additional moonlight functions[45] that promote the angiogenesis, gene-expression, nucleic acids stability, tumor chemoresistance[46], protection against caspase-independent cell death[47], metastatic potential, motility[48] and cell cycle dysregulation[49]. Indeed, parallel to its primary function in the glycolysis, GAPDH plays also a key role in multiple cellular activities such as nuclear *t*RNA export, maintenance of DNA integrity through its telomeric association, mRNA stability, control of gene transcription and translation, endocytosis and exocytosis, cytoskeletal organization and apoptosis.[43, 50-52] Notably, GAPDH represents the primary cell sensor for oxidative stress, hypoxia and ischemia.[53-55] In addition to regulating the enzymatic glycolytic activity of GAPDH, post-translational modification such as S-nitrosylation affects the cellular compartmentalization of GAPDH and thus define its non-glycolytic functions. Indeed, whereas glycolytic activity is due to cytosolic GAPDH, post-translational modification as well as protein-protein interactions may induce the translocation of GAPDH from cytoplasm to the nucleus, mitochondria or extracellular microenvironment, thus directing GAPDH to its non-glycolytic functions.[56] Whereas preclinical evaluation of some GAPDH inhibitors has clearly shown a correlation between the anticancer activity and the inhibition of glycolysis, less is known about how these inhibitors may affect the non-glycolytic functions of GAPDH, although in some cases they have been used as tools to study specific moonlight functions and this represent an attractive area of research.[57, 58] Due to its widespread diffusion, GAPDH is associated with numerous pathological conditions. The pronounced duality of GAPDH behavior in physiological and pathological conditions inspired Sirover et al.[53] to describe it as "Janus of the cell", in reference to the Roman god of beginnings and endings, usually depicted of having two faces.

In addition to cancer research area, targeting GAPDH represents a promising perspective for the treatment of bacterial infections[32] and tropical parasitic diseases caused by protozoa such as *Trypanosoma*[59], *Plasmodium*[60, 61] and *Leishmania*[59], for which the development of new drugs acting on new targets is a topic

of major interest and importance since there are large numbers of patients involved and the current therapies suffer from many drawbacks such as excessive toxicity and the recurrent problem of drug resistance. These microorganisms rely almost entirely on glycolysis for ATP production and consequently are much sensible to GAPDH inhibitors. Lastly, there are increasing evidences about the involvement of GAPDH in neurodegenerative disorder pathogenesis such as Alzheimer's disease, Parkinson's disease, Huntington disease[62] and diabetes.[63, 64]

In this review, we report an overview of the available agents able to covalently inhibit GAPDH, ranging from non-specific metabolites and pharmacological tools to actual drug-like compounds endowed with promising therapeutic features.

2. Development of GAPDH Covalent Inhibitors

Among all the known covalently modulated targets, the most prevalent are enzymes, which represents almost 90% of the cases and the most modified residues are cysteine and serine, followed by co-factors, lysine, threonine and others.[1] Notably, cysteine is underrepresented in the human proteome (2.26%)[65], and its thiol function is highly nucleophilic. Therefore, it represents a viable residue to be selectively targeted by moderately electrophilic warheads. Despite the overwhelming majority of the freshly approved cysteine-targeting covalent drugs present the acrylamide Michael acceptor warhead (Ad_{NM}), a recent study by Ábrányi-Balogh et al.[66] highlighted that there are many other suitable warheads, which rely on different reaction mechanisms such as nucleophilic-addition (Ad_N), addition-elimination (Ad-E), nucleophilic substitution (S_N) and oxidation (Ox). Their findings also suggest that cysteines surrounded by different microenvironments, and hence having different nucleophilicities, in some cases exhibit different susceptibility to the various types of warheads. In particular, the reactivity of S_N warheads increases with increasing of the nucleophilicity of the targeted cysteine, while for acrylate-based electrophiles (Ad_{NM}) no correlation was observed. Therefore, S_N warheads should be taken into consideration for the rational design of covalent inhibitors targeting nucleophilic cysteines, like the ones present in the catalytic site of GAPDH. Among the various S_N warheads, α-haloacetamides, acyloxymethyl ketones, halomethyl ketones and activated aryl halides have been widely exploited for cysteine-targeting inhibitors despite their medium-high intrinsic reactivity.[67] Thus, it is clear that the warhead should be carefully selected on a case by case basis during each drug discovery program to achieve tailored reactivity and specificity for the targets. Interestingly, according to the Pearson HSAB model[68], soft electrophiles such as α,β-unsaturated carbonyls react faster with soft nucleophiles as Cys sulfhydryl groups than His and Lys.[69, 70] Moreover, the catalytic Cys of GAPDH is present in a pK_a-lowering microenvironment -the catalytic triads- and its sulfhydryl group is highly nucleophilic at physiological pH ($pK_a \approx 6.0$). Therefore, the side-chain thiol can be easily deprotonated to thiolate, which is a soft nucleophile endowed with higher nucleophilic index ($\omega-$) and higher reactivity than the protonated thiol.[70] These kinetic differences can be exploited for tuning the warhead selectivity towards the desired amino acidic target.[71]

2.1 UNSPECIFIC ENDOGENOUS MODULATORS AND XENOBIOTICS AS BIOCHEMICAL TOOLS

As already mentioned, GAPDH is a homotetrameric enzyme, and each subunit is featured with three cysteine residues, one of which is involved in the catalytic mechanism. The functionality of this enzyme is deeply modulated by a number of endogenous signals, which impact on its activity in both physiological and pathological conditions, and interestingly, most of the post-translational modifications are directed to the cysteine residues.[19, 72] Moreover, covalent pharmaceutical tools are used to turn off the activity of GAPDH and slow down glycolysis in specific biological assays.[73-76] Therefore, a systematic analysis of these endogenous and xenobiotic covalent ligands can provide important information for the identification of potential electrophilic chemical motifs to guide the rational design of selective and effective GAPDH covalent inhibitors.

2.1.1 Endogenous modulators and synthetic analogues

Dimethyl fumarate is a synthetic analogue of fumarate, a Krebs cycle intermediate, and its use is approved for the treatment of psoriasis[77] and multiple sclerosis.[78] This compound is metabolized *in vivo* to monomethyl fumarate[79] and both these agents exert an immunomodulatory effect. Dimethyl fumarate and monomethyl fumarate can bind covalently, through the so-called succination reaction, to specific cysteine residues of certain proteins including KEAP1, with the sequential activation of the anti-inflammatory transcription factor Nrf-2. Recent studies demonstrated that these fumarate derivatives also covalently bind GAPDH both at the active site (Cys-152 in human) and at non-catalytic Cys-156 and Cys-247, leading to inhibition of the enzyme. This inhibition of GAPDH is an irreversible process with a biphasic behaviours, thus k_{inact}/K_i values were measured for both the fast (k_{inact}/K_i dimethyl fumarate = 18.3 M^{-1} s^{-1}, MMF = 5.9 M^{-1} s^{-1}) and slow (k_{inact}/K_i dimethyl fumarate = 10.9 M^{-1} s^{-1}, monomethyl fumarate = 3.2 M^{-1} s^{-1}) phases.[80] Analysis of the crystal structure of monomethyl fumarate-bound human GAPDH (PDB code: 6IQ6) revealed that the steric hindrance of the linked inhibitor avoids the correct binding of NAD^+ cofactor.[81] Moreover, dimethyl fumarate and monomethyl fumarate showed strong inhibition of GAPDH and interruption of aerobic glycolysis in activated immune cells (mouse peritoneal macrophages), hampering cell metabolism and causing a reduction of the inflammatory process.[80] Remarkably, also endogenous fumarate was found to modify the catalytic cysteine of GAPDH covalently. Incubation of rabbit muscle GAPDH with 0.5 mM fumarate for 24h led to 20% loss of activity, while complete inhibition was achieved with 500 mM of fumarate for the same time.[63]

Figure 2. Reaction of GAPDH with different α,β-unsaturated endogenous compounds

The endogenous immunomodulator Itaconate is a metabolite of Aconitate, another Krebs cycle intermediate, and it is directly involved in the regulation of macrophage function.[82] Its precise mechanism of action is still unclear. It was shown that Itaconate and its membrane-permeable surrogate 4-octyl itaconate (4-OI) are able to directly alkylate cysteine residues of KEAP1, promoting the antioxidant and anti-inflammatory response mediated by Nrf-2.[83, 84] Interestingly, likewise fumarate and its analogues, they were also shown to covalently inhibit GAPDH in RAW264.7 murine macrophage cell line. In particular, in these cells, the α,β-unsaturated warhead alkylates the thiol of Cys-22 (via Michael addition, decreasing the enzyme activity,[85] whereas the catalytic cysteine was not affected.

In a context for the study of the catalytic mechanism of GAPDH, Castilho and co-workers[17] synthesized a small set of phosphorylated compounds. In particular, derivatives **1** and **2**, which are respectively structurally similar to itaconate and GAP, displayed inhibitory properties towards *T.cruzi* GAPDH through covalent inhibition of the catalytic cysteine. Kinetic studies showed that **1** possess a 6-times faster inactivation rate of *Tc*GAPDH compared to **2**, therefore it was exploited as pharmacological tool for crystallographic experiments. Notably, it was found that compound **1** can bind to the thiol both via Michael addition on the α,β-unsaturated

system and via Addition-elimination on the ester function. Together with further related researches[86], this work gave an important contribution for the understanding of the GAPDH catalytic flip-flop mechanism.

Figure 3. Possible reaction of GAPDH with compounds **1** and **2**

Furthermore, GAPDH is also strongly inhibited by two well-known endogenous 4-hydroxy-2-alkenals: 4-hydroxy-2-nonenal (HNE, IC_{50} after 1h = 50.0 µM) and 4-hydroxy-2-hexenal (HHE, IC_{50} after 1h = 39.1 µM), commonly associated to ageing, oxidative stress and pathological conditions and originated from lipid peroxidation processes.[86] These highly reactive α,β-unsaturated aldehydes covalently attach to the Cys, His and Lys residues located in the surface of the enzyme in an apparently indiscriminate way, without modifying the inner catalytic cysteine.[87] Following these modifications, GAPDH loses its activity and afterwards, it is recognized and degraded by Cathepsin G.[86] Conversely, 2-hexenal and 2-nonenal are not GAPDH inhibitor, suggesting that the 4-OH group of HNE and HHE is essential for GAPDH inhibition and further degradation.[32] It has to be mentioned that compounds such as 4-HNE can modify nucleophilic residues of various cellular proteins and subsequently impair their functions, thus such compounds should be regarded only as biochemical tools and can drive the design of more specific compounds.[88]

Methylglyoxal (MGO) is another side-product of several physiopathological pathways associated to age-related diseases. This α-oxoaldehyde with two adjacent reactive carbonyl groups can inhibit GAPDH through covalent adduction on unspecific residues.[89]

2.1.2 α,β-unsaturated xenobiotics

Similarly to what described for endogenous modulators, GAPDH enzymatic activity can be affected by reactive xenobiotics presenting α,β-unsaturated carbonyls such as Acrylamide (ACR)[90], Methyl Vinyl Ketone (MVK) and Acrolein. These toxicants are originated from environmental pollution and can also be found as dietary contaminants and in the cigarette smoke.[91] In particular, these compounds can form Michael adducts with diverse relevant thiols in the intracellular environment, including the well-studied catalytic cysteine residue of GAPDH leading to complete inactivation of the enzyme. Martyniuk et al.[92] performed an in-depth study of GAPDH inhibition kinetics by these three structurally related toxicants, and they found a strong connection between the calculated electrophilic index (ω) and the k_{inact}/K_i values. In fact, among the compounds, ACR presented a moderate electrophilic reactivity and lower k_{inact}/K_i, and it is more selective for the nucleophilic catalytic Cys. Interestingly, their results suggest that the marked preference for cysteines over other alkylable residues as His and Lys can be explained by the higher calculated electronic compatibility between the thiol group and the α,β-unsaturated warheads. In a separate work, Acrylonitrile (AN), another vinyl toxicant, was incubated with GAPDH and here too it was observed a remarkable selectivity for the Michael addition on the catalytic cysteine, leading to an effective inactivation of the enzyme.[93]

Compound	Structure	ω (eV)	k_{inact}/K_i (M^{-1} s^{-1})
Acrolein		3.82	297

MVK		3.38	128
ACR		2.61	0.053
AN		-	9.2

Table 1. Comparison of electrophilic indices and inhibition kinetic constants of the principal α,β-unsaturated xenobiotics

The vesicant agents ethyl vinyl sulfone and divinyl sulfone were found to inhibit GAPDH with a mixed mechanism composed by a reversible oxidative component due to Cys oxidation (similarly to H_2O_2) and an irreversible one, caused by Cys alkylation. Notably, these two sulfones were selective for GAPDH over other cysteine residues-containing enzymes such as Isocitrate Dehydrogenase and Glucose-6-Phosphate dehydrogenase.[94] Lastly, GAPDH is covalently inhibited at the cysteine residues by the air pollutant *N*-ethyl maleimide (NEM), one of the components of diesel-exhaust airborne particles[95] that is also employed in several biochemical studies for cysteine targeting.[96]

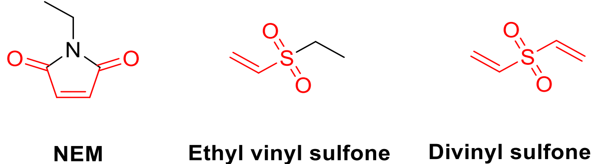


Figure 4. Chemical structures of *N*-ethyl maleimide, ethyl vinyl sulfone and divinyl sulfone

Despite these toxicologically relevant chemicals are obviously too small to be regarded as selective tools, the comprehension of their time-dependent reactivity towards GAPDH cysteine residues provides valuable insight for further implementation of these chemical motifs in novel and effective GAPDH inhibitors.

2.2 α-HALOMETHYLCARBONYL COMPOUNDS

Among the non-endogenous covalent GAPDH inhibitors, another broad category is represented by α-halomethylcarbonyl compounds which undergo S_N2 reactions with nucleophilic side-chain residues of the enzyme.[71] The known acylpeptide hydrolase inhibitor acetylleucine chloromethyl ketone (ALCK) was found to induce GAPDH degradation followed by apoptosis in U937 leukemia cell line.[97] The mechanism was deeply explored, and prior to the decrease of GAPDH, it was observed a potent and irreversible inhibition of the enzyme activity.[98] ALCK unspecific binding to GAPDH induces a significant conformational change that targets the enzyme for subsequent proteolysis, in a similar way to what mentioned before for HNE/HHE.[86] In the past decades, several tools bearing the α-halomethylcarbonyl warhead were developed and used for kinetic studies. The electrophilic reactivity of these compounds is directly proportional to the leaving group properties of the halogen (I > Br > Cl > F). Indeed, Iodoacetamide and Iodoacetate react indiscriminately with almost all the available cysteine residues, while their Bromo- and Chloro- analogues are more suitable for ligand design.[99, 100] Bromo-acetyl phosphonate (BAP) and Chloro-acetyl phosphonate (CAP) are irreversible inhibitors of GAPDH, displaying impressively high rate constants, respectively 5.4×10^4 M^{-1} s^{-1} and 2.8×10^4 M^{-1} s^{-1} against yeast-holoenzyme. BAP rate constant was also assessed against human GAPDH (3.5×10^4 M^{-1} s^{-1}) and other isoforms.[101] Despite these α-haloacetyl phosphonates are less reactive than iodoacetamide against model thiols, their close similarity to the endogenous substrate GAP allows them to selectively alkylate the catalytic cysteine of GAPDH. 3-halo pyruvic acids are the most relevant GAPDH covalent inhibitors featured with the α-halomethylcarbonyl warhead, and the entity of their pharmacological effect is directly related to their electrophilic reactivity.[102] In particular, 3-Bromopyruvate (3-BP) is a well-known halogenated compound capable of “pyruvilation” of reactive thiols of a variety of enzymes.[103] Notably, its structure is superimposable to BAP. The mechanism of action was deeply investigated in recent years[104, 105],

showing that the proapoptotic effect of 3-BP is the result of the combinatorial inhibition of a plethora of catabolic enzymes [106, 107] supported by its ability to interfere with cellular GSH and mitochondrial functionality, causing oxidative stress.[108, 109] Since its activity is mainly focused on the block of the glycolytic pathway, more specifically by inhibiting GAPDH[110-112], 3-BP was extensively studied as anticancer agent for targeting the Warburg effect.[113-115] Moreover, 3-BP has a preferred uptake by cancer cells[116], which endows it with a catastrophic potential over their bioenergetic machinery. Despite the alkylating properties of 3-BP are commonly associated with non-selective toxicity[111], it showed an effective mechanism of action and an outstanding selectivity towards cancer cells.[117-120] In addition to its anticancer properties, as expected from GAPDH covalent inhibitors, 3-BP also exhibits a promising antiprotozoal activity on *Trypanosoma brucei* and *Toxoplasma gondii* [121, 122] and antifungal activity on different species.[123, 124] Two ester derivatives of 3-BP were studied as anticancer agents. The propyl ester (**3**) exhibited micromolar activity against colon cancer cells and excellent selectivity for GAPDH[112], while the perillyl ester (**4**) has been recently patented and validated as GAPDH inhibitor.[125] The latter showed a cytotoxic potency higher than the co-administration of 3-BP and perillyl alcohol, which possess itself anticancer properties[126] and *in vivo* studies on mice showed high selectivity for cancer cells without organ toxicity or other significant side effects.[125] Moreover, its ability to freely cross the membranes without specific carriers reduce the chances of the insurgence of a resistance mechanism.

ALCK **Iodoacetate** **Iodoacetamide**

BAP: X = Br
CAP: X = Cl

3-BP: R = H
3: R = nPr
4: R =

Figure 5. Chemical structures of GAPDH inhibitors presenting the α-halomethylcarbonyl warhead

2.3 EPOXIDES

The epoxide group (oxirane) is a warhead prone to be attacked by nucleophile thiols (S_N2) undergoing ring-opening. Its reactivity arises from its electrophilic nature and its ring strain and can be modulated to avoid non-specific interactions with GSH and other relevant thiols. The ability of arene oxides to covalently inhibit GAPDH was proved back in 1978.[127] However, arene oxides are generally carcinogenic or mutagenic molecules able to bind non-specifically to macromolecules. On the contrary, very interesting examples of epoxide-containing molecules can be found among natural compounds, which display high selectivity for GAPDH owing to their structural similarity with the substrate GAP. Similarly, selective inhibitors can be designed by coupling the epoxide ring to a substrate mimicking moiety.

2.3.1 Epoxy-phosphate compounds

Compounds **6-8** are GAP analogues bearing the epoxide group as electrophilic warhead and they can covalently inhibit the enzyme. Notably, **6** shows a 20-fold higher reactivity for the enzyme than for GSH.[128] The reaction of glycidol phosphate (**6**) with GAPDH is not preceded by a reversible binding step, differently from the behavior of the dephosphorylated analog glycidol (**5**). This can be explained by the higher tendency of GAPDH to react with the epoxide as a consequence of the interaction with the phosphate moiety, which induces a conformational change of the enzyme.[128] Willson et al.[129] synthesized phosphate esters **7** and **8** to enhance the cellular permeability of **6**, which displayed no significant activity on *T. brucei* and these compounds were tested against rabbit muscle GAPDH (*Rb*GAPDH) and *Tb*GAPDH to evaluate their inhibitory parameters. **6**, **7** and **8** act as irreversible covalent inhibitors of GAPDH through alkylation of the catalytic cysteine, and remarkably the hydrophobic derivatives **7** and **8** exhibited an even higher affinity for GAPDH compared to **6**. Docking studies revealed that the phosphoric heads of the inhibitors occupy the P*i* site, conversely to GAP behavior. Notably, the (*R*) and (*S*) enantiomers displayed a similar reactivity against the enzyme, suggesting a high flexibility of the catalytic pocket for the reaction with the epoxides. Replacing the phosphate moiety with phosphonates group leads to a complete activity loss.[130] Since all the phosphates are active, while the corresponding phosphonates have no activity, it seems that a critical distance of 4.2 Å between the phosphorous atom and the reactive epoxide must be respected.[130]

5

6: R = H
7: R = Et
8: R = Bn

Figure 6. Chemical structures of GAPDH inhibitors presenting the epoxy-phosphate structure

2.3.2 Koningic acid (or Heptelidic acid or Avocettin)

Avocettin was first isolated from the cultures of *Anthostoina avocetta* by Sandoz researchers[131] and cultures of *Chaetomium globosum*, *Trichoderma viride* and *Gliocladium virens* by Sankyo scientists and referred as "Heptelidic Acid" during a screening program for new antibiotics,[132] while "Koningic Acid" (KA) was isolated afterwards by Endo from *Trichoderma koningii*.[133] Sequential structural studies revealed Heptelidic acid and KA to be the same substance.[133, 134]. The structure of KA was resolved by spectroscopic methods[132] and confirmed by X-ray crystal structure analysis.[134] KA biosynthetic pathway remained partially unclear until 2019, when its biosynthetic gene cluster was identified using gene disruption and expression analysis.[135] A total synthesis of (±)-Heptelidic acid was published by Danishefsky et al. in 1988[136] while an enantiospecific total synthesis[137] was provided in 1997. Moreover, a scalable and flexible synthesis of racemic Koningic Acid was recently published.[138] Studies on the mechanism of action of KA revealed a specific inhibition of GAPDH[133] over other metabolic enzymes, that is confirmed also by a recent comparative metabolomic study.[46] The GAPDH inhibition first occurs through the formation of a reversible complex between KA and GAPDH and then with an irreversible inhibition of the enzyme activity.[139] Further observations have confirmed that GAPDH is inhibited by the formation of a covalent bond between the epoxide moiety of KA and the thiol group of catalytic Cys.[140] KA indeed structurally resembles the features of GAP: it presents an epoxide ring as alkylating moiety, an H-bond acceptor group represented by a lactone carbonyl to mimic the polar hydroxyl group of GAP and a carboxylic acid group capable of ionic interaction as the phosphate of the substrate. The distances between the three functions are superimposable in the two structures. Thus, the reason for the inhibition of GAPDH mediated by KA could be found in its structural relation to GAP.[141] Different isoforms of GAPDH from various sources exhibit different relative sensitivities to KA. These differences have been explained by a comparison of their amino acid sequences.[142] In particular, sensible isoforms show an unusual high similarity in the S-loop domain adjacent to the catalytic triad, suggesting that this region can determine the susceptibility of GAPDH to KA. Remarkably, KA-producing strain of *T. koningii* M3947 was found to

express a KA-resistant GAPDH isozyme under conditions of KA production.[143] Since its GAPDH inhibitory activity has been extensively studied in the past, KA has become a well-known GAPDH inhibitor, and there are many examples of its application in biochemical and biomedical researches.[144-147] KA was tested *in vivo* on mice and was found to be bioavailable, tolerable at certain doses, and capable of inducing acute and dynamic changes in the glycolytic network in tumors.[46] Furthermore, it exhibited biological activity against microorganisms such as anaerobic bacteria[132] and malaria parasites[148] both *in vitro* and *in vivo*. Moreover, it has been extensively studied as an anticancer agent exploiting the Warburg effect through GAPDH irreversible inhibition followed by ATP depletion and malignant cells death.[43, 149] Cytotoxicity of KA was tested against several cell lines of cancer[149, 150] showing low micromolar IC_{50}s, while healthy cell lines were insensitive to the inhibitor.[149] KA has recently been tested on 60 cancer cell lines demonstrating various grades of sensibility.[46] In 2015 Rahier et al.[150] performed some SAR studies on KA derivatives, since its pharmacokinetic parameters were unsatisfactory, and *in vivo* activity against lung cancer xenograft model A549 was minimal. The study showed that the electrophilic moiety is fundamental for the antiproliferative activity; indeed the corresponding diol derivative was found to be inactive, while the chlorohydrin analog exhibited almost the same activity of KA.[151] The carboxylic group is also essential for KA activity and it can be substituted only with a suitable ester.[150] In case of easily hydrolyzable esters, it cannot be ruled out the possibility of restoring the -COOH function *in vitro* and *in vivo*. In contrast, stable derivatives such as methyl amide and methyl ester possess a lower activity compared to KA.

Figure 7. Koningic Acid

2.3.4 Pentalenolactone (or Arenaemycin E)

Arenaemycin E was first isolated from cultures of *Streptomyces arenae* in 1957 by Celmer et al.[152] and subsequently by Takeuchi and co-workers, in 1969 and called Pentalenolactone (PL).[153] Finally, it was isolated by a group of Upjohn's chemists during screening programs for antimetabolites with antitumor activity and X-ray analysis confirmed Arenaemycin E and PL to be the same substance.[154, 155] The biosynthesis of PL was studied in considerable detail,[156-158] and its total synthesis was accomplished in 1978-1979 by Danishefsky et al.[136, 159] PL, similarly to KA, has been identified as an irreversible covalent GAPDH inhibitor reacting with the catalytic cysteine in the active site, due to its close similarity with the superimposable structure of GAP.[141, 160-162] The PL producing microorganism (*Streptomyces arenae* TÜ469) was found to express two different GAPDH isozymes: a PL-sensitive isoform and an inducible, PL-insensitive isoform, responsible for the self-resistance.[163-165] The pharmacodynamic effect of PL is attributable only to the selective inhibition of GAPDH, since it showed no activity on different enzymes involved in glucose uptake, glycolysis and intermediary metabolism both in *E. coli* and *B. subtilis*.[161] PL inhibitory activity was assessed on isolated GAPDH from a variety of sources[166], including *T. brucei*, rabbit muscle, *Saccharomyces cerevisiae*, *B. Stearothermophilus* and *E. coli*, showing low- and sub-micromolar IC_{50}s.[167] Furthermore, PL showed to be active against a range of microorganisms, including Gram-positive and Gram-negative bacteria, pathogenic and saprophytic fungi and protozoa and it was found to inhibit the replication of DNA viruses, including HSV-1 and HSV-2, the causal agents of *Herpes simplex*.[141] Activity in the µM range was proved against erythrocytes (rat), hepatocytes (rat), Ehrlich-ascites tumor cells (mice), *P. vinckei* infected erythrocytes (mice), *Saccharomyces cerevisiae*[160] and *T. brucei*.[168, 169] The emerged data allow some SAR considerations on PL structure, in order to evaluate whether it is possible to enhance its drug-like properties and activity towards GAPDH *in vitro* and *in vivo*. Arenaemycin C (chlorohydrin) shows an inhibitory behavior similar to PL; the same was observed for KA and the

correspondent chlorohydrin.[161] In this case, esterification of the carboxylic function with a methyl group led to a 10-fold reduction of activity with respect to the PL benzylamine salt.[141] Interestingly, the co-metabolite pentalenic acid, which possesses the conjugated double bond but not the epoxide moiety, is completely ineffective as GAPDH inhibitor.[162] This is another confirmation that the epoxide ring is the actual electrophilic warhead, and that the covalent inhibition occurs via S_N2 epoxide opening and not via Michael addition to the α,β-unsaturated system. These findings are also supported by the fact that the completely saturated derivative tetrahydropentalenolactone is a potent irreversible inhibitor of GAPDH.[162]

Figure 8. Pentalenolactone

2.4 QUINONES

Quinones[170-172] and naphthoquinones[171, 173] are electrophilic warheads able to undergo Michael addition reaction with reactive cysteine thiol groups. Indeed, the reaction of quinones with thiols is kinetically favored with respect to amines.[174] Many quinone compounds are coming from the oxidative metabolism or autoxidation of catechol and hydroquinone endogenous compounds,[175, 176] natural molecules[177-180] and xenobiotics[181]. Their phenolic form, after converting to quinone, becomes capable of reactivity towards Cys residues of GAPDH, often unspecifically. Notably, without the combination with a proper recognition moiety, the 1,2-quinone warhead exhibited a lack of selectivity in chemical proteomic studies.[170]

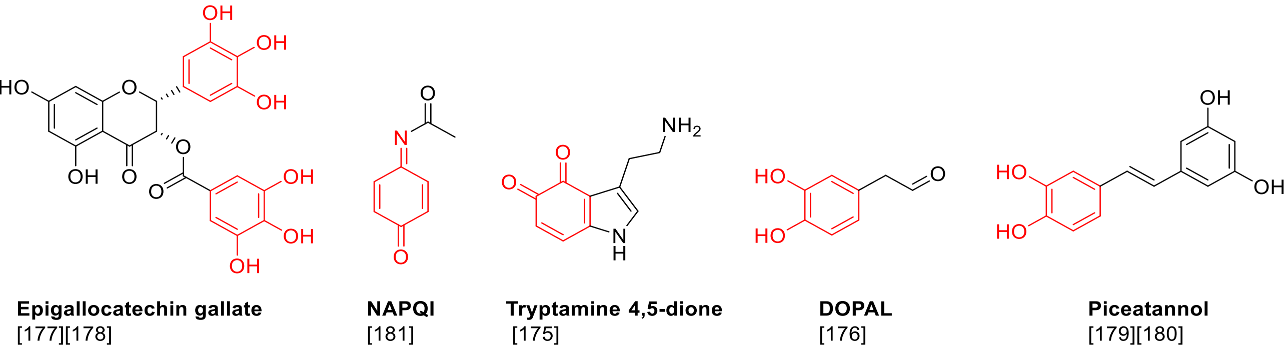


Figure 9. Chemical structures of unspecific GAPDH inhibitors presenting the quinonic warhead

2.4.1 2-Phenoxy-1,4-naphthoquinones and antraquinones

Despite small molecules do not reach selectivity for the GAPDH covalent inhibition, more complex compounds such as 2-phenoxy-1,4-naphthoquinones and 2-phenoxy-1,4-anthraquinones are successful examples of selective compounds against GAPDH. These compounds arise from the natural 1,4-naphthoquinone Lapachol, which showed activity against *Trypanosoma* and *Leishmania* without cytotoxic effect on human cells.[182] In-depth SAR studies were performed, and several active derivatives emerged.[183, 184] Although the mechanism of action of quinones is generally related to their unspecific ability to generate free radicals[185, 186] and to interfere with the mitochondrial respiratory chain[187-190], recent findings demonstrate that the principal effect of 2-phenoxy-1,4-naphthoquinones is the covalent inhibition of the

catalytic cysteine of GAPDH, which was confirmed by MALDI-TOF analysis of undigested *Pf*GAPDH after incubation with 2-phenoxy-1,4-benzoquinone.[191] The GAPDH inhibition is accompanied by consequent ATP depletion and cell death.[191, 192] However, these compounds exert their cytotoxic action through a multi-target mechanism involving ROS production and therefore interfering with the electron transport chain.[183] Moreover, some of these compounds showed good activity and selectivity on cells that relies majorly on glycolysis for energy production as parasites (*Trypanosoma*, *Leishmania*, *Plasmodium*) and cancers cells.[183, 188] The covalent modification of the active site cysteine (Cys165 in *Tb*GAPDH) is also supported by covalent docking experiments of 2-phenoxy-1,4-napthoquinones with the catalytic pocket of the enzyme. In particular, the most active compounds displayed a very similar binding mode, which is consistent with the covalent binding of the warhead to the catalytic cysteine.[183] Covalent inhibition can occur either via Michael addition or addition-elimination with the phenoxy displacement from the reactive thiol of GAPDH. Both these mechanisms have already been reported for phenoxybenzoquinone derivatives.[193] Moreover, the alkylating properties of these derivatives were tested against two different cysteine proteases that were unaffected by their presence, thus suggesting their potential selectivity towards GAPDH within cysteine-dependent enzymes.[191] To evaluate a further possible selectivity between different GAPDH isoforms, 2-phenoxy-1,4-naphthoquinone was incubated with human, *T. brucei*, *P. falciparum* and *A. thaliana* GAPDH orthologs. *Pf*GAPDH exhibited the fastest inactivation kinetic, followed by *h*GAPDH, while *At*GAPDH and *Tb*GAPDH were the slowest reacting isoforms.[191]

Figure 10. Different possible reactions of GAPDH with 2-phenoxy-1,4-naphthoquinones and 2-phenoxy-1,4-anthraquinones

The same authors also reported a series of quinone-coumarin hybrids (Figure 11) with a dual-targeted antitrypanosomal profile. Notably, in these derivatives GAPDH inhibition occurs through covalent Cys binding only to the quinone moiety, whereas the coumarin portion does not react.[194] In particular, despite the presence of an α,β-unsaturated moiety, the conjugated phenyl ring determines a detrimental effect on the warhead reactivity as Michael acceptor. A similar lack of reactivity was observed for other coumarin derivatives such as Chalepin, which behaves as a GAPDH inhibitor but with a non-covalent mechanism.[195-199]

Figure 11. A dual targeted phenoxynaphthoquinone-coumarin hybrid

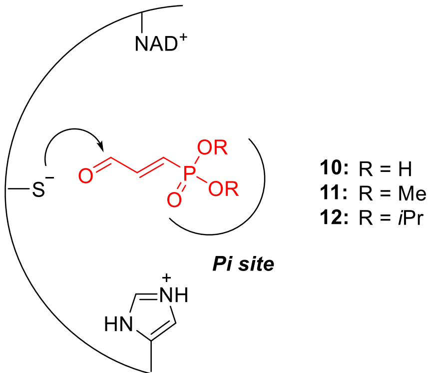
2.5 α,β-UNSATURATED COMPOUNDS

As mentioned before, some small α,β-unsaturated compounds both from endogenous and exogenous sources, including fumarate, itaconate, acrylamide, acrolein, acrylonitrile and others, exhibited the peculiar ability to inhibit GAPDH strongly through covalent binding to cysteine residues. Although these molecules

are not suitable as such, due to their small dimensions, lack of selectivity and of drug-like properties, their α,β-unsaturated warhead represents a viable starting point for the development of more selective and drug-like GAPDH inhibitors.

2.5.1 α,β-Unsaturated phosphonates

Starting from the structure of the endogenous substrate GAP, Willson et al.[129] synthesized a small set of α,β-unsaturated phosphonates able to inhibit *Tb*GAPDH and *Rb*GAPDH through the covalent binding exclusively on the catalytic Cys, as validated with Cys-titration experiments.[130] The covalent mechanism of action was confirmed with NMR studies, by monitoring the phosphorous chemical shifts before and after incubation with the enzymes.[129] Remarkably, although these inhibitors are expected to undergo Michael addition as commonly reported for α,β-unsaturated compounds, it was found that the aldehyde function is responsible for the covalent reaction with the cysteine thiol. This mechanism can be explained by the optimal distance between the aldehyde and the phosphonic head. In particular, as corroborated by modelling studies[130], the latter can establish a convenient interaction with the P*i* site, with the consequent positioning of the reactive aldehyde close to the nucleophilic thiol, similarly to what mentioned before for the epoxide analogues. However, since attempts to co-crystallize *Tb*GAPDH complexed with **11** have failed, a precise binding mode of these derivatives within the catalytic pocket is not available.[129] Despite the high structural similarity between the trypanosome and human GAPDHs, **10** and **11** exhibited some preference for the parasitic enzyme, displaying higher k_i/K_{inact} values.[129] The compounds were also tested in vitro against cell cultures of *T. equiperdum*. The ionizable compound **10** was found to be inactive, probably due to cell permeability issues, while **11** and **12** showed a complete inhibition of parasite multiplication (LD_{100}) respectively at 10 and 0.3 µM, which is probably due to the complete and instantaneous inhibition of the glycolysis, as suggested by the observed interruption of oxygen uptake and ATP production by the trypanosomes.[129, 130] Remarkably, these activities are much higher than the ones observed for the two well-known trypanocidal drugs pentamidine (LD_{100} = 2 mM) and (difluoromethyl)ornithine (LD_{100} = 100 µM) under the same conditions.[129] The selectivity of this set of compounds was not deeply investigated against other glycolytic enzymes.



10: R = H
11: R = Me
12: R = *i*Pr

Figure 12. Proposed binding pose and reaction of compounds **10-12** with GAPDH

2.5.2 Isochaihulactones

The root extracts of *Bupleurum scorzonerifolium*, commonly used in traditional Asian medicinal preparation for the treatment of influenza, fever, malaria, cancer and menstrual disorders[200], were found to possess an in *vitro* and *in vivo* anticancer activity imputable to the inhibition of tubulin polymerization, resulting in induction of cell cycle arrest at G2/M, with consequent apoptosis.[201, 202] The main active compounds were identified in the racemic mixture of (*Z*)-isochaihulactone (*R*,*S*-**13**), which also exhibited a strong induction of apoptosis both *in vitro*, against several cell lines and *in vivo* on A549 xenograft mouse.[203, 204] In particular, the (*S*) enantiomer was found to be 7-10 times more active than its enantiomer, showing a 1 µM activity towards

three different cancer cell lines.[205] In a very recent study using molecular probes, the non-catalytic Cys-247 of *h*GAPDH was identified as one of the main targets of (*S*)-**13**. The glycolytic activity of *h*GAPDH, which is mediated by the catalytic Cys-152, was not impaired. Thus, the GAPDH-mediated cytotoxicity should be related to its other cellular roles and moonlight functions. Indeed, it was demonstrated that the selective alkylation of Cys-247 controls the "ON/OFF" switch of apoptosis through non-canonically nuclear GAPDH translocation. This novel mechanism of action highlights the importance of GAPDH as a target for tumor treatment, since this mechanism bypasses the standard apoptosis-resistant route of MDR cancers.[205] GAPDH covalent inhibition occurs via Michael addition on the α-methylene lactone group, which was already described as a thiol reacting feature.[206] This warhead presents a finely modulated reactivity due to the electron-donating properties of the conjugated trimethoxyphenyl moiety; therefore, unspecific reactions with thiols were only sluggishly observed.[205] Moreover, the selectivity of (*S*)-**13** for Cys-247 over the much more nucleophilic catalytic Cys is due to the higher affinity of the recognition moiety for a site of the enzyme different from the catalytic pocket. Several SAR studies were carried out on the structure of this dibenzylbutyrolactone ligand and interesting findings emerged, which are resumed in Figure 13.[204, 205, 207]

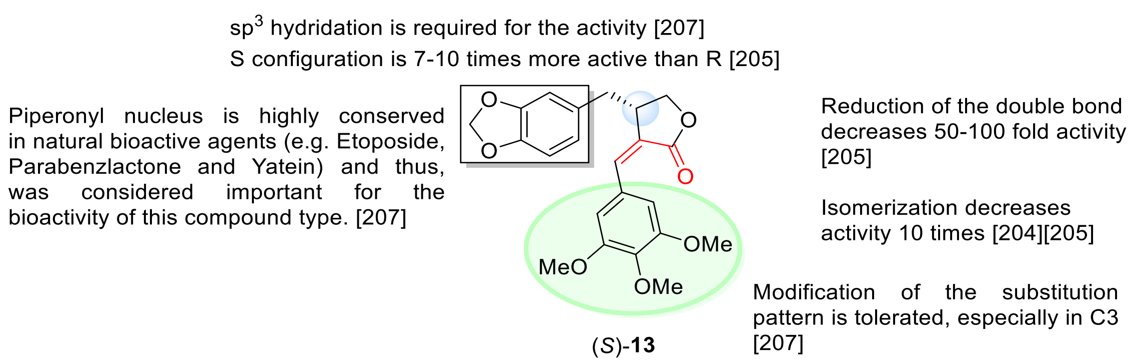


Figure 13. Structure-Activity Relationship of compound (*S*)-**13**

2.6 3-BROMO ISOXAZOLINES

α-Amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid, better known as Acivicin, is a natural compound isolated from *Streptomyces sviceus* broths by Hanka et al.,[208] which exhibits a broad set of anticancer, antibacterial and antiparasitic activities.[209, 210] Acivicin presents the quite singular 3-chloro-4,5-dihydroisoxazoline nucleus as an electrophilic warhead, which can covalently bind to activated nucleophilic enzymatic residues including Ser, Thr and Cys.[211] Due to its structural similarity with the natural aminoacid L-Gln, Acivicin behaves as a covalent irreversible inhibitors of several glutamine amidotransferases (GATs), characterized by a conserved catalytic triad consisting of cysteine, histidine and glutamate. The mechanism of action was elucidated in chrystallographic studies using Acivicin co-crystallyzed with the glutaminase domain of *T. brucei* CTP synthetase.[212] Acivicin undergoes nucleophilic attack from the catalytic Cys residue leading to the formation of a tetrahedral oxyanion, with a sp^3-hybridized C3, followed by the release of chloride and restoration of the sp^2 C3. Interestingly, the corresponding synthetic brominated analogue was shown to be more active than Acivicin, when tested against *T. brucei* CTP synthetase[213] due to the better leaving group properties of bromine. Recently, the 3-Bromo-Δ^2-isoxazoline nucleus has gained attention as an emerging electrophilic scaffold, which was successfully used in the design of covalent inhibitors of different classes of Cys-containing enzymes.[213-224] Moreover, the comparison of the proteome targets of Chloro and Bromo-isoxazoline revealed that the first achieve weaker labelling with the same targets compared to the bromide counterparts.[225] 3-Bromo-Δ^2-isoxazoline represents an ideal warhead with a relatively low reactivity towards nucleophiles unless orientated at a distance and angle favorable for the reaction. This allows the design of appropriate ring substituents to achieve selectivity towards specific residues, avoiding the risk of uncontrolled reactivity against off-target proteins or physiologically relevant

thiol-containing peptides, like glutathione. In addition, the five-membered nitrogen-oxygen containing ring could establish favorable interactions with the typical polarized environment of the catalytic pocket of the enzyme.[226] More recently, 3-Bromo Acivicin (3-BA) was tested as antimalarial and showed sub-micromolar activity against *Plasmodium falciparum* cell cultures (IC_{50} = 0.34 μM).[227] Since *P. falciparum* relies only on glycolysis for energy production, the activity of 3-BA as a potential inhibitor of *Pf*GAPDH was assessed and indeed it showed a moderate activity towards *Pf*GAPDH (k_{inact}/K_i = 0.7),[227] which was not observed for Acivicin.[225, 228] Structural optimization of 3-BA was aimed at reducing the structural similarity with L-Gln, so to decrease the affinity for GAT enzymes, and, in parallel, to increase the affinity for the catalytic pocket of *Pf*GAPDH (Figure 14).[14] As a result, new potent *Pf*GAPDH inhibitors were identified, with sub-micromolar activities against both Chloroquine sensitive and resistant strains of *P. falciparum*. Interestingly all derivatives showed low toxicity against the human endothelial cell line HMEC-1, displaying selectivity indices for *P. falciparum* > 30 (compounds **14** and **16**), > 175-250 (compound **15**) and > 350-400 (compound **17**). Interestingly, these compounds displayed a characteristic selectivity mechanism towards *Pf*GAPDH over human GAPDH. Specifically, the alkylation of the catalytic site of one of the four identic monomers of *h*GAPDH by 3-Bromo-Δ^2-isoxazolines induces a conformational rearrangement that completely masks the remaining three sites from further alkylation by the inhibitor preserving, therefore, three-quarters of the activity. On the contrary, this mechanism is not observed for *Pf*GAPDH. In that case, incubation with the inhibitor leads to complete inactivation of the enzyme.[229]

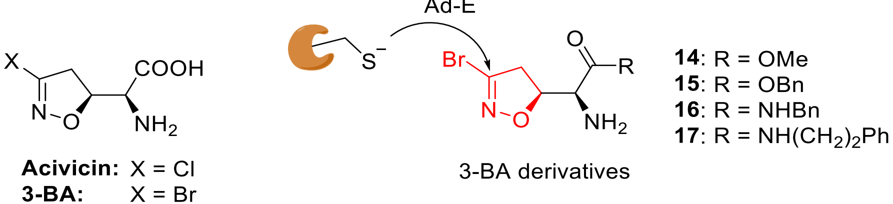


Figure 14. Proposed reaction of 3-Bromo-Δ^2-isoxazolines with GAPDH

2.7 ACYLOXY NITROSO COMPOUNDS

We have already mentioned the possibility of *S*-nitrosylation of the catalytic Cys by HNO, which also occurs in the intracellular environment rich of thiols such as GSH.[42, 230, 231] Indeed, treatment of rabbit muscle GAPDH with the HNO precursors Angeli's salt (AS) and Piloty's acid causes the formation of a disulfide bridge (Cys149−Cys153) in the active site of GAPDH and sulfinamides at Cys244 and Cys281.[232, 233] Acyloxy nitroso compounds (**18-24**) are a new group of NO-containing compounds. [233, 234] These compounds can inhibit GAPDH via covalent modification of the cysteine residues. However, also Nitrosobenzene (PhNO), which does not release HNO, inhibits GAPDH, meaning that the electrophilic nitroso group can itself behave as an electrophilic warhead, undergoing nucleophilic addition by GAPDH cysteines. For these compounds, the HNO release represents only the second step of the mechanism, therefore its rate is directly subordinate to the rate of the preceding ester hydrolysis.[233, 235]

Figure 15. Proposed reaction of acyloxy nitroso compounds with GAPDH

Thus, based on their ester hydrolysis rate, acyloxy nitroso compounds can be divided in those which rapidly release HNO, merely acting as HNO-donors (e.g. compound **20**) and those which are stable enough to behave as electrophiles and bind to the catalytic cysteine leading to covalent inactivation of GAPDH (e.g. compound **21**). In particular, the former caused the formation of Cys sulfinamides as the predominant product, the latter generated sulfinic acids and *N*-substituted sulfinamides and are more potent GAPDH inhibitors.[233, 235] Compounds with intermediate reactivity as **23** and **24** were found to inhibit GAPDH through both mechanisms. Remarkably, treatment of GAPDH with acyloxy nitroso derivatives and PhNO led to the formation of a reversible disulfide bond between Cys149 and Cys153.[233] Thus, the acyloxy nitroso warhead possess attractive features due to its properties of reacting with cysteine residues: tuning their NO-donating properties in favor of their electrophile reactivity towards nucleophilic thiols makes them promising candidates for drug development as GAPDH inhibitors. Indeed, the reactivity of this warhead can be modulated by selecting an appropriate ester substituent, and SAR studies should be encouraged in order to develop selectivity towards GAPDH. Since acyloxy nitroso compounds are GAPDH inhibitors, even without NO-donating properties, they are potential anticancer agents,[233] while NO-donors have been traditionally used for their vasodilating properties.[234]

2.8 AROMATIC THIOLS

NAC (*N*-(phenoxyacetyl)-L-cysteine and NMC (*N*-((*R*)-mandelyl)-L-cysteine) act as covalent *Rb*GAPDH inhibitors by selectively forming an oxidized disulfide bridge with the catalytic Cys, in a similar way to what described for GSH.[236] The inhibition mechanism, which was elucidated with docking studies, showed that NAC and NMC establish non-covalent interaction with the NAD^+ binding site, placing the mercaptan group at a favorable distance to form the disulfide bridge with the catalytic Cys of GAPDH. Interestingly, while NMC and GSH can inhibit the enzyme only when NAD^+ is displaced, NAC is able to bind even in the presence of NAD^+, showing a much greater inhibitory activity. Moreover, it was demonstrated that the binding of the inhibitors and subsequent modification of sulfhydryl groups occurs in only two monomers of the tetramer.[236] Despite the intriguing and novel mechanism of action of these ligands, no additional information concerning their target selectivity are reported.

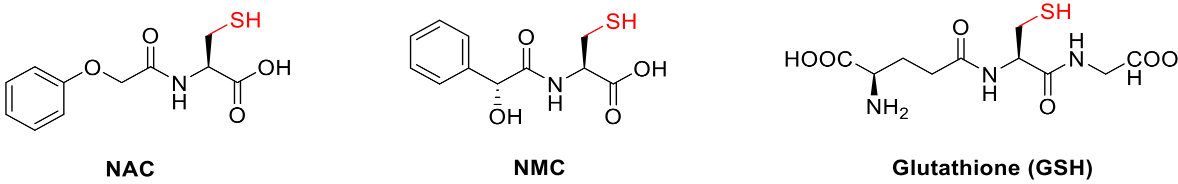


Figure 16. Chemical structures of GAPDH inhibitors presenting the sulfhydryl group as a warhead

3. SUMMARY AND PERSPECTIVE

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an emblematic moonlight protein, which not only plays a pivotal role in the glycolytic pathway[237], but also exhibits multiple intriguing activities in different subcellular compartments including membrane fusion, endocytosis, ER/Golgi trafficking, mRNA stability, tRNA export, DNA integrity, nuclear transcriptional regulation and, most importantly, is involved in the apoptotic process.[53, 56, 60, 238] In particular, some of these moonlight functions are directly related to the immune system regulation,[31, 80] and survival and progression of the tumor cells.[53] Moreover, due to its well-characterized role in glycolysis GAPDH is rate limiting for the Warburg effect in cancer cells.[46, 147, 239] Furthermore, GAPDH is also a rate-limiting enzyme in those cells that mainly, if not only, rely on glycolysis for energy production, such as protozoan parasites.[34, 59, 122, 188, 227] For these reasons, GAPDH represents a polyhedral target and its inhibition, especially if achieved with a covalent mechanism, can be a valuable approach for the treatment of several pathological conditions. Covalent inhibitors are powerful and performant tools in drug discovery, since their ability to strongly bind to the target, primarily on cysteine residues, can lead to an irreversible inhibition that lasts until the re-synthesis of the protein. The recent re-emergence of covalent inhibitors has boosted the research of covalently-druggable target with the final goal to reach new therapeutic potential. Each subunit of GAPDH presents three cysteine residues, one of which is involved in the catalytic mechanism, which are amenable for post-translational modifications and xenobiotic covalent binding. Therefore, we reported a systematic overview of the endogenous and exogenous covalent ligands able to affect the activity of GAPDH, focusing on the different warheads, on their mechanism of actions and their effects. These sophisticated inhibitors are composed of two parts: (i) a warhead, that is the electrophilic centre capable of reacting covalently with the target, decorated with (ii) a recognition moiety responsible for the affinity for the target. To achieve a selective mechanism of action and to direct the warhead exquisitely on the chosen alkylation site, both the two parts must be considered during drug design. Warhead selection is a nontrivial task and is typically based on the inhibition mechanism (Ad_N, Ad_{NM}, Ad-E, S_N, Ox) and on the reactivity required for the interaction, considering the HSAB theory, in order to increase its greed for the specific nucleophile target. Moreover, to avoid uncontrolled off-target interactions and related toxicity, the warhead reactivity must be finely tuned. It can be influenced by the presence of electron-donor or electron-withdrawing groups. The recognition moiety has an essential function for the interaction with the target, establishing non-covalent interaction with the side chains of the amino acidic residues of the selected pocket, orientating the warhead at a distance and angle favorable for the nucleophilic attack on the desired site. With this strategy, as we have shown in this review, it is possible to achieve a complete inhibition of the enzyme also exploiting warheads with low intrinsic electrophilic reactivity, and therefore with lower toxic potential. On the other hand, highly reactive warheads such as strained epoxides and alpha-halo derivatives can be made selective for a given target when coupled to a highly specific recognition moiety, and their reactivity can be mitigated by proper chemical modifications, preventing their toxicity and unspecific mechanism. In the present review, we have presented several classes of covalent GAPDH inhibitors, ranging from endogenous regulators, small reactive molecules, synthetic compounds and natural products, presenting different warheads and different mechanism of action. Unfortunately, a systematic comparison of the selectivity profile of these inhibitors cannot be performed due to the lack of activity-based protein profiling studies. A comparative metabolomics analysis identified koningic acid as a specific GAPDH

inhibitor, compared to compounds such as 3-Bromopyruvate and Iodoacetate.[46] For compounds such as those belonging to the 3-Br-isoxazoline series, although the possibility that these compounds may interact with additional targets was not ruled out, low cytotoxicity against human endothelial cells (HMEC-1) was observed with very good selectivity indices for *P. falciparum*, up to > 350-400 for compound **17**.

Altogether, the data summarized in this review indicate as more promising those compounds inspired by nature, since the increased complexity of the recognition moieties may guarantee the desired selectivity. Thus novel selective covalent GAPDH inhibitors may be designed by combining features from the natural world with synthetic chemical moieties. The design approach may include placing a novel warhead on an already established recognition moiety able to mimic the enzyme substrate, or by a structure-based design of the recognition moiety to support an already validated warhead.

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