Glutaminase regulation in cancer cells: a druggable chain of events

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Metabolism is the process by which cells convert relatively simple extracellular nutrients into energy and building blocks necessary for their growth and survival. In cancer cells, metabolism is dramatically altered compared with normal cells. These alterations are known as the Warburg effect. One consequence of these changes is cellular addiction to glutamine. Because of this, in recent years the enzyme glutaminase has become a key target for small molecule therapeutic intervention. Like many oncotargets, however, glutaminase has a number of upstream partners that might offer additional druggable targets. This review summarizes the work from the current decade surrounding glutaminase and its regulation, and suggests strategies for therapeutic intervention in relevant cases.

Introduction

During the past decade, cancer metabolism has drawn a significant amount of attention as a target for therapeutic intervention. This is because most cancer cells experience the Warburg effect, described in 1924 by the German biochemist Otto Warburg who observed that cancer cells undergo a high rate of glycolysis and lactic acid fermentation, even under normoxic conditions [1]. The Warburg effect has since been intensively studied, with numerous reports characterizing various aspects of the altered metabolism that it describes. One interesting facet, from a chemotherapeutic point of view, is that, in a large number of cases, cells undergoing a Warburg effect exhibit a marked dependence upon glutamine, to the extent that these cells are referred to as being 'glutamine addicted' [2]. Glutamine addiction arises from the need for extracellular glutamine to be consumed for anaplerotic input in the citric acid cycle, which accounts for the majority of the bioenergetic needs of normal (nontransformed) cells. The key gatekeeper of this input is the enzyme glutaminase.

Glutaminase exists as two forms in humans: one that was originally named kidney-type glutaminase (KGA) derived from the *GLS1* gene and a second called liver-type glutaminase (LGA) derived from the *GLS2* gene [3]. Whereas LGA is expressed primarily in the liver, KGA has been found to be ubiquitously distributed [4]. We will thus focus on KGA, because its ubiquitous distribution renders it more likely to be relevant to a number of cancer types. Indeed, KGA has been shown to be upregulated in tumors from diverse systems such breast, lung, cervix, brain and B cells, with glutaminase inhibition having slowed the proliferation of these cancer cell lines [2,5–8]. KGA exists as two splice variants that differ only in their C-terminal regions, with the longer form retaining the acronym KGA and the shorter form being called glutaminase C (GAC) [9]. GAC has been detected in a wide variety of cancer cell lines in culture [2,10].

KGA and GAC are generally believed to localize to the mitochondria, although the exact intramitochondrial localization is still under debate [11,12]. The primary function of the glutaminase enzymes is to catalyze the hydrolysis of L-glutamine to L-glutamate, the latter being generally unable to enter the mitochondria directly. As L-glutamate is formed it is converted to α-ketoglutarate by the enzyme glutamate dehydrogenase (GDH). This product can then be utilized directly in the citric acid cycle, leading to energy and building block production. One other important function of glutamine metabolism is to provide precursors for glutathione production, which helps to maintain the oxidative status of cells. Indeed, glutaminase has been directly linked to redox balance in cancer cells [13–15].

In their inactive states, KGA and GAC exist primarily as dimeric species. *In vitro*, KGA or GAC can be activated via the addition of inorganic phosphate, which is thought to stimulate the formation of an active tetramer [16,17]. Little is currently known about the

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regulation of either enzyme in cells, but research performed during the past several years has begun to uncover a number of pathways that might lead to their expression and activation. Additionally, a minimum of ~40 mM phosphate is required to activate glutaminase *in vitro*, whereas serum phosphate levels tend to be ~1 mM, further suggesting that, in cells, glutaminase is activated by mechanisms involving something other than inorganic phosphate [18,19]. As is the case for many signaling proteins implicated in oncogenic transformation, the upstream regulators of glutaminase activity could serve as excellent drug targets, with some in fact already being the focus of drug discovery efforts.

This review summarizes several recently investigated mechanisms by which glutaminase activity can be modulated via pharmacological agents and examines the most recently developed small molecule inhibitors against KGA and/or GAC and their regulators. Specifically, we examine two recently discovered glutaminase inhibitors [968 and *bis*-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES)], proceed to a discussion about upregulators of glutaminase [c-Myc, nuclear factor κB (NF-κB), signal transducer and activator of transcription 1 (STAT1) and the rapidly accelerated fibrosarcoma-mitogen-activated protein kinase kinase-extracellular signal-regulated kinase (Raf–MEK–ERK) pathway], examine some potential negative regulators [anaphase-promoting complex/cyclosome (APC/C) and Lon Protease (LON)] and finally discuss several recent discoveries in the brain, where glutamine metabolism serves the additional purpose of operating as a neurotransmitter. The scope of this review does not permit a similar in-depth analysis of metabolic pathways downstream of KGA and GAC. However, glutamine and glutamate metabolism downstream of KGA and GAC in cancer is extensively reviewed elsewhere, and the reader is invited to consult these sources for such information [20–22].

Direct inhibition of KGA and GAC

For many years, the predominant drug used to target KGA directly was 6-diazo-5-oxy-L-norleucine (DON; Fig. 1). DON acts as an irreversible glutamine-competitive inhibitor. Although effective against glutaminase, DON is not selective and has several verified targets [23].

During the past decade, several new small molecules have been discovered that inhibit KGA and its splice variant GAC. One of these molecules is 968 (Fig. 1), which was discovered by our laboratory and was shown to be an allosteric regulator of GAC [2,24]. The inhibitory potential of 968 has been described in a number of cancer cell lines in culture, as well as in a mouse xenograft model [2]. Owing to the hydrophobic nature of the molecule, it has been difficult to use in animal models and almost all studies to date have been in cell culture. Our most recent report has described the SAR surrounding the 'hot-spot' region of the molecule: the halo-benzene ring, which was initially determined to be crucially important for inhibitory potency. We have determined that the electronic nature of the substituents on the ring is relatively unimportant but that they must impart steric bulk perpendicular to the plane of the ring to show a significant inhibitory effect against GAC.

Others have utilized 968 in a variety of studies, demonstrating its potency against GAC and KGA. Simpson *et al.* conducted studies examining metabolically sensitive epigenetic markers, focusing upon histone H3 and histone H4 acetylation and trimethylation and the effects of these modifications upon a number of cancer-related genes [25,26]. Upon treatment with 968, they found that cells tended to exhibit, for example, enhanced H4 lysine 16 acetylation but that histone deacetylase activity was not significantly impacted overall. These investigators further showed that oncogenes such as Akt and ErbB2 were substantially downregulated, thus suggesting that glutaminase inhibition might be a more effective epigenetic therapy than the use of histone deacetylase inhibitors, which tend to have a broader impact on cells.

More recently, Huang *et al.* utilized 968 while testing the hypothesis that glutamine metabolism via glutaminase in cancer cells is more responsible for the control of intracellular pH (via ammonia release) than for providing inputs to the citric acid cycle [6]. Although this goes against the established doctrine, the investigators provide intriguing evidence that the modulation of cellular acidity represents at least one important function of glutamine metabolism, showing that glutamine withdrawal was far less lethal to HeLa or MCF-7 cell lines if growth media maintained at pH 7.3 was used rather than growth media maintained at pH 6.3. 968 was then used to show that cell growth was preferentially inhibited at lower pH. These results fail to account for studies showing that cell lines resistant to glutamine withdrawal gain sensitivity if glutamine synthetase inhibitors are added. Glutamine synthetase would, by producing glutamine, presumably cause ammonia to be consumed as an outcome of enzymatic activity, and acidify the cellular environment. Nevertheless, these findings still pose an interesting secondary mechanism regarding glutamine utilization in the tumor environment [27].

968 has resisted enzyme co-crystallization efforts, leaving its exact mechanism of action unknown. Kinetic studies have shown that 968 acts as an allosteric inhibitor, and does not compete with glutamine; moreover, mutagenesis experiments suggest that it binds in a pocket formed between the N and C termini of a GAC dimer (Fig. 2). Additionally, we have recently developed fluorescent reporter group assays that enable us to show that 968 binds directly to GAC (unpublished). GAC is activated by undergoing a dimer-to-tetramer transition that can be stimulated *in vitro* by 50–100 mM inorganic phosphate [16,17]. Under conditions where GAC has been activated before 968 addition the ability of the drug to inhibit enzyme activity is severely compromised, whereas if the drug is added to GAC just seconds before activating the enzyme with inorganic phosphate the drug exhibits its full inhibitory effect. This finding probably explains at least part of the difficulty in forming a GAC – 968 co-crystal, because crystallization trials are typically performed at protein concentrations that would drive GAC to an active tetramer by mass action (i.e. even without the addition of inorganic phosphate). We hypothesize that GAC undergoes relatively large structural changes upon its activation and inhibitor binding, and that 968 cannot bind the activated enzyme. To the best of our knowledge, 968 has not yet been examined as a potential inhibitor of the LGA isoform of glutaminase.

The second important glutaminase inhibitor to be described during the past few years is BPTES (Fig. 1), a symmetrical molecule specific for KGA over the LGA isoform. BPTES has been shown via X-ray crystal structures to bind at the interface where two KGA