A tumor agnostic therapeutic strategy for Hexokinase 1 Null/Hexokinase 2 positive cancers

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Financial disclosure: no conflict of interest.
ABSTRACT: Since Warburg’s observation that most cancers exhibit elevated glycolysis, decades of research have attempted to reduce tumor glucose utilization as a therapeutic approach. Hexokinase (HK) activity is the first glycolytic enzymatic step; despite many attempts to inhibit HK activity, none has reached clinical application. Identification of HK isoforms, and recognition that most tissues express only HK1 while most tumors express HK1 and HK2, stimulated reducing HK2 activity as a therapeutic option. However, studies using HK2 shRNA and isogenic HK1+HK2- and HK1+HK2+ tumor cell pairs demonstrated that tumors expressing only HK1, while exhibiting reduced glucose consumption, progressed in vivo as well as tumors expressing both HK1 and HK2. However, there exist HK1-HK2+ tumor subpopulations among many cancers. shRNA HK2 suppression in HK1-HK2+ liver cancer cells reduced xenograft tumor progression, in contrast to HK1+HK2+ cells. HK2 Inhibition, and partial inhibition of both oxidative phosphorylation and fatty acid oxidation using HK2 shRNA and small molecule drugs, prevented human liver HK1-HK2+ cancer xenograft progression. Using human multiple myeloma xenografts and mouse allogeneic models to identify potential clinical translational agents, triple therapies that include antisense HK2 oligonucleotides, metformin and perhexiline prevent progression. These results suggest an agnostic approach for HK1-HK2+ cancers, regardless of tissue origin.

Key words: hexokinase 2, precision medicine, glycolysis, metabolism, antisense oligonucleotide
INTRODUCTION

Glucose metabolism, leading to the oxidation of its carbon bonds, results in stored energy in the form of ATP. In mammals, the end product of glucose metabolism can be lactate, when oxygen is insufficient, or carbon dioxide upon full oxidation of glucose via respiration in the mitochondria. In most tumors both glucose utilization and lactate production are dramatically augmented, even in the presence of oxygen. This process of increased aerobic glycolysis is known as the Warburg effect (1).

While the Warburg effect is required for tumor growth (2), the function of the Warburg effect remains controversial (3). First, the Warburg effect supports rapid ATP synthesis to provide tumor cells with a selective advantage when competing for shared and limited energy resources in the tumor microenvironment (4,5). Second, the increased glucose consumption can be used as a carbon source for anabolic processes, including the generation of nucleotides, lipids, and proteins needed to support cell proliferation (6). In addition, glucose can be utilized to generated NADPH from the oxidative branch of the pentose phosphate pathway, or through pyruvate metabolism, to generate NADPH in the TCA cycle. Glucose can also be used through fatty acid oxidation to generate NADPH, which meets rapidly the demands of proliferating cancer cells for reducing equivalents (6-8). Third, the Warburg effect confers direct signaling functions to cancer cells both by modulating protein kinase networks as well as by protein posttranslational modifications (9,10). Last but not least, in addition to the cell-intrinsic functions, the Warburg effect may provide an advantage for cancer cell growth in a multicellular environment via acidification of the microenvironment to facilitate
immunosuppressive M2 macrophage polarization (11), and limiting glucose availability to tumor infiltrating lymphocytes (12,13).

Targeting the Warburg effect for cancer treatment has been investigated for nearly a century. However, because of the conserved nature of the glycolytic pathway in normal tissues, global systemic inhibition of glycolysis results in adverse effects that have made this approach of limited value (14,15). Thus it seems clear that selective inhibition of cancer-driven glycolysis will be required to exploit this metabolic pathway for clinical cancer therapy.

Development of the positron-emitting glucose analogue $^{18}$F-fluorodeoxyglucose ($^{18}$F-FDG) (16) and positron emission tomography (PET) technology (17) led, in the late 1970’s and early 1980’s, to early clinical applications of $^{18}$F-FDG-PET to study, non-invasively, glucose metabolism in the brain (18) and heart (19), and in cancer (20). In addition to the increased aerobic consumption of glucose observed in most cancers, increased hexokinase activity is a major contributor to the resulting increased conversion of $^{18}$F-FDG to $^{18}$F-FDG-6-phosphate ($^{18}$F-FDG-6P) in these tumors. Subsequent development of $^{18}$F-FDG PET clinical imaging has played a major role in initial cancer diagnoses, detection of metastases, monitoring of disease and therapeutic response, and in identification, selection and refinement of therapeutic protocols.

The hexokinases (HKs), the first enzymes committed to glycolysis, convert glucose to glucose-6-phosphate (G6P). There are four conventional HK isoforms, HK1/2/3/4, encoded by four different genes (21). Most adult tissues express only HK1. Muscle and adipose tissue use HK2 for glycolysis, liver and pancreatic β cells express HK4 (also called glucokinase) and do not express HK1 or HK2. HK3 is inhibited by
physiological concentrations of glucose. A new HK isoform, hexokinase domain containing 1 (HKDC1), was recently discovered; HKDC1 likely phosphorylates glucose in maternal metabolism in pregnancy (22), but was reported to be associated with cancer prognosis (23). While HK4 has only one kinase structural active site domain, HK1, HK2 and HK3 have two such structural domains (N- and C-domains). The conventional HK isoforms share sequence similarity in their enzymatic active site domains. HK4 has only one kinase structural active site domain; this site possesses enzymatic activity. Both HK1 and HK3 have an N-terminal structural site domain without enzymatic activity and a C-terminal active site domain with enzymatic activity. HK2 has both an N-terminal active site domain with enzymatic activity and a C-terminal active site domain with enzymatic activity. Consequently, HK2 is more catalytically efficient than the other HK isoforms. In addition, both HK1 and HK2 can be located on the mitochondrial surface as a result of binding to voltage-dependent anion-selective channel protein 1 (VDAC1); this mitochondrial association provides privileged access for these enzymes to the ATP synthesized in the mitochondria to support their kinase activity. While HK1 has a strong binding affinity for VDAC1, HK2 binds less avidly to VDAC1 and alternates between cytoplasmic and mitochondrial-bound states (24).

In contrast to normal tissues, many tumors express both HK1 and HK2. Although elevated HK activity as a driver of tumor glycolysis was reported in the late 1970's (25), it was approximately 30 years later that HK2 expression was identified in most cancers (26). The elevated and relatively restricted expression of HK2 in most cancers and the tolerability of a global HK2 gene deletion in adult mice (27) suggested HK2 as a promising cancer-restricted target for cancer therapy (27-30).
HK1^HK2^ tumor cells are resistant to HK2 inhibition or elimination.

While most normal tissues express only HK1, the majority of cancers express both HK1 and HK2. The assumption has been that the added expression of HK2 contributes to the increased glycolysis and the tumorigenesis observed in cancer cells. A number of laboratories, working on this assumption, investigated the ability of HK2 shRNAs (shHK2s) to modulate cancer cell viability and proliferation in culture, and tumor progression in xenograft mice. Data from mouse xenograft tumor models for human breast (27), lung (27), pancreatic (28) and prostate (29) cancer, and glioblastoma (30) were interpreted to suggest that shHK2 knockdown slowed or inhibited the progression of these tumors, and to suggest that selective pharmacologic HK2 inhibition has the potential to be a near-globally effective cancer therapeutic approach. Moreover, global HK2 deletion in adult mice is tolerated (27). These data stimulated intense interest in the development of selective HK2 inhibitors for cancer therapy (31-33).

Our recent study (34) directly compared the roles of HK1 and HK2 in tumor cell growth and xenograft progression. For this enquiry we used cancer cell lines reported in previous studies (27-30) which express both HK1 and HK2, as well as shHK2 sequences reported in previous studies (27-30) and a more effective inducible shHK2 developed in our own laboratory (34). In contrast to previous reports (27-30), we found that neither the proliferation of these HK1^HK2^ cancer cells in cell culture, nor the growth of xenograft tumors derived from these cells, was affected by shHK2 knockdown (34).

In addition, we used an orthogonal approach to examine the role of both HK1 and HK2 in tumor progression in xenograft models. Using CRISPR Cas9-mediated
HK1 or HK2 knockout to generate isogenic cohorts of HK1^KOHK2^+, HK1^+HK2^KO and HK1^+HK2^+ cancer cell lines, we confirmed that HK1^+HK2^+ cancer cells can tolerate either HK1 or HK2 inactivation (34). Xenograft tumors derived from parental lung cancer H460/HK1^+HK2^+ cells, and isogenic H460/HK1^KO and H460/HK2^KO cells all grew at comparable rates in culture, and progressed at comparable rates when examined as xenograft tumors (34). These findings indicate that there is redundancy in HK activity in cancer cells, and that HK1 is sufficient to maintain cancer cell proliferation in cell culture and tumor growth in vivo, in the absence of HK2. In agreement with our observations, HK2 is redundant for the function of activated T cells, which express both HK1 and HK2, indicating a compensating effect of HK1 in maintaining adequate glycolysis (35,36).

Isogenic tumor cells that lack HK1 (HK1^KOHK2^+) or HK2 (HK1^+HK2^KO) can progress at equivalent rates as individual xenografts (34). However, the presence of HK2 might confer a growth advantage for tumor cells and provide a mechanism by which HK1^+HK2^+ tumor cells predominate if HK2^+ cells emerge in a tumor environment that is initially predominantly HK1^+HK2^-. To examine this possibility, we mixed isogenic HK1^+HK2^+ H460 cells and HK1^+HK2^+ H460 cells at a 1:9 ratio, and determined whether the proportions of the two tumor cell types changed as these tumor cell mixtures progressed as xenografts (Fig. 1A and B). However, the percentages of HK1^+HK2^- and HK1^+HK2^+ cells in the resulting xenograft tumors remained the same during tumor progression and were the same percentages as those of the HK1^+HK2^- and HK1^+HK2^- cells in the initial mixed cell population injected subcutaneously into the mice (Fig. 1C). These data provide further support for the conclusion that HK2 is dispensable for HK1^+HK2^+ tumor progression.
Neither cell proliferation nor xenograft progression for HK1\(^+\)HK2\(^+\) cancer cells were affected by HK2 shRNA knockdown or CRISPR knockout (34). However, glucose consumption and lactate production in HK1\(^+\)HK2\(^-\) cancer cells were significantly reduced when compared to the isogenic HK1\(^+\)HK2\(^+\) cells (34). In addition, \(^{18}\)F-FDG sequestration was significantly reduced in HK1\(^+\)HK2\(^-\) isogenic tumor xenografts when compared to their parental HK1\(^+\)HK2\(^+\) tumors (34). These findings further demonstrate that there is redundant glycolytic activity in HK1\(^+\)HK2\(^+\) cancer cells, both in culture and \textit{in vivo}.

**HK1 null, HK2 positive (HK1\(^-\)HK2\(^+\)) tumor cells are sensitive to HK2 inhibition.**

Analysis of RNA sequencing (RNA-Seq) data of cells from the Cancer Cell Line Encyclopedia (CCLE) dataset revealed that subsets of cancers from a variety of tissues of origin express only HK2 and do not express HK1 (33,34,37). While HK1\(^-\)HK2\(^+\) cancer cell line subsets exist in cancers from a variety of tissues of origin in the CCLE collection, multiple myeloma and liver cancers are the most enriched in members that lack HK1 expression; 54% of the multiple myeloma cell lines and 38% of the liver cancer cell lines are HK1\(^-\)HK2\(^+\). In addition to these CCLE dataset results, an analysis of the Cancer Genome Atlas (TCGA) liver tumor RNA-Seq data revealed that 83% of the HK2 expressing liver tumors were HK1 negative (37). Such information on HK1 and HK2 expression for other collections of TCGA tumor types would be of great value. However, extending the RNA-Seq analysis to datasets of other TCGA tumor types is challenging because of the HK1 expression by the presence of normal tissue in the tumor samples. HK1 presence from normal tissue might confound these bulk tumor mRNA level measurements. In contrast, normal hepatocytes express only
HK4/glucokinase, and do not express HK1 or HK2. Consequently, contamination of liver tumor samples with HK1 mRNA signal from normal liver tissue is not a problem, in contrast to tumor samples from other tissues.

Without the support of HK1-driven glycolysis, HK1^HK2^+ cancer cells are susceptible to major growth inhibition when HK2 is targeted (34). Compared to HK1^HK2^+ cells from liver cancer, bladder cancer, and multiple myeloma, which tolerated the effect of shHK2 knockdown with no apparent inhibition of cell proliferation, the proliferation of HK1^HK2^+ cells from the same cancers was significantly reduced by shHK2 knockdown (34).

Consistent with our suggestion that HK1 and HK2 expression in cancer cells provide redundant protection from loss of function for either of these HK isoforms, in an unbiased genome-wide screen for essential genes for cancer cell growth or survival, using the HK1^HK2^+ chronic myeloid leukemia (CML) cell line KBM7, neither HK1 nor HK2 was identified as an essential gene (38). In contrast, and consistent with our suggestion that HK2 is targetable in cancer cells lacking HK1, HK2 was identified as an essential gene in a genome wide screen of Jiyoye cells, a lymphoma cell line with a low HK1 level (38).

To validate and extend the observation that targeting HK2 inhibits proliferation of HK1^HK2^+ cancer cells but not HK1^HK2^+ cancer cells, we utilized our H460 lung cancer cell isogenic HK2 genetic model. While the parental H460 HK1^HK2^+ xenograft lung tumors were resistant to the effect of shHK2 knockdown on tumor growth inhibition (Fig. 1D), the growth of xenograft tumors derived from isogenic H460/HK1^HK2^+ cells, established by CRISPR Cas9 HK1 deletion, was significantly reduced by shHK2 knockdown (Fig. 1E) (34). In addition, xenograft tumors derived from naturally existing
HK1^−HK2^+ cancer cells of liver cancer and multiple myeloma were significantly suppressed by HK2 knockdown (33,34,37). These results provide evidence that the clinical efficacy of targeting HK2 is likely to be limited to HK1 deficient cancers. Furthermore, the threshold level of HK1 that is sufficient to support cancer cell growth remains unclear. Future studies will be needed to determine the threshold level of HK1 that can support tumor sustaining glycolysis activity in individual cancer types when HK2 is inhibited. These data could help to identify cancers with HK1 expression below such thresholds and extend HK2-targeted cancer therapy to these sub-groups.

**Targeting HK2 with clinically translatable selective HK2 inhibitors**

Because of the conserved nature of the glycolytic pathway in normal tissues, pan-inhibitors of HK isoforms have considerable adverse effects that make global HK inhibition of limited value for cancer treatment (14,15). For example, glucose insufficiency causes damage and death of brain cells (39). shHK2 is ineffective in preventing proliferation in culture and progression in xenografts for HK1^+HK2^+ cancers, but can slow both growth in culture and xenograft tumor progression of HK1^−HK2^+ cancers (**Fig. 1D and E**). Consequently, for therapeutic purposes selective pharmacologic inhibition of HK2 will be ineffective in treatment of HK1^+HK2^+ cancers, but may be effective for treatment of HK1^−HK2^+ cancers. While HK2 is a potential target for this subset of cancers, it has been challenging to selectively drug this HK isoform, due to its extremely polar active site and the high structural similarity between HK2 and the HK1 isozyme present in most normal tissues (31).
In an effort to develop a selective HK2 inhibitor for cancer treatment, GlaxoSmithKline performed a high throughput screen of a 2-million compound library, using purified recombinant HK2 (31). The identified hit compound, a non-selective HK inhibitor, was further optimized using co-crystal structure- and structure-activity-analysis guided modifications, resulting in HK2 inhibitors with a greater than 100-fold selectivity for HK2 over HK1 in HK enzymatic activity assays (31). However, neither HK2 selectivity, nor on-target efficacy in cell culture or in animal xenograft models, have been reported for these HK2 inhibitors.

Behar et al (32) reported a small molecule, Comp-1, which preferentially detaches HK2, but not HK1, from the mitochondria surface docking protein VDAC1. As a consequence, the close proximity of HK2 to ATP produced in mitochondria is impaired and HK2 activity is selectively reduced. In a cell-free microscale thermophoresis assay, Comp-1 detached HK2 from VDAC1 with an IC50 value of 0.092 μM. In cell culture, Comp-1 selectively reduces mitochondrion-bound HK2 with an IC50 value of 0.8 μM. Comp-1 inhibits colony formation and cell proliferation in a panel of 57 cell lines from multiple cancer types, including both HK1^HK2^+ cell lines HepG2 and HL60, as well as HK1^HK2^+ cell lines HCT116, MiaPaca2 and MDAMB231. The HK1 and HK2 protein levels for these cell lines have previously been reported (37). Comp-1 on-target potency has not been evaluated in an isogenic HK1^HK2^+, HK1^HK2^+, and HK1^HK2^- cell system. Comp-1 in the form of a topical ointment was effective in treating UVB-induced skin damage in a mouse model. Given that Comp-1 can inhibit proliferation of HK1^HK2^+ tumor cells, while our data demonstrate that HK1^HK2^+ cells are not susceptible to HK2 targeted growth inhibition either by shHK2 or by HK2 deletion
(33,34,37), we suggest that Comp-1 efficacy in killing HK1^HK2^ tumor cells must include some off-target effect(s) in addition to its modulation of HK2 interaction with mitochondrial VDAC1.

Antisense oligonucleotide (ASO) therapeutic agents have the advantage that they selectively degrade specific RNA targets and prevent the synthesis of the corresponding proteins, even among closely related isoforms, based on differences in mRNA target sequences. Consequently, targeted pharmacological ASOs can discriminate in the inhibition of synthesis for closely related proteins with shared biological activities. A number of ASO therapeutic agents have been approved for clinical use (40,41) and more are currently under clinical evaluation (42).

Using Ionis Pharmaceuticals’ latest Generation 2.5 Chemistry technology, we developed selective ASOs for human HK2 (hHK2 ASOs). Pharmacologic HK2 inhibition efficacy for HK1^HK2^ cancers was examined for these hHK2 ASOs, using human multiple myeloma cells as another disease model (33). Multiple myeloma is the cancer type with the highest HK1^HK2^ population percentage in the CCLE dataset (33,34). In cell culture, hHK2 ASOs entered multiple myeloma cells via free uptake, without using any transfecting agents. The hHK2 ASOs were able to substantially diminish hHK2 protein levels in multiple myeloma cells at 2.5 μM or lower concentrations without affecting HK1 protein levels. As a consequence, hHK2 ASOs selectively inhibited the proliferation of human HK1^HK2^ multiple myeloma cells, without significant growth inhibition in human HK1^HK2^ multiple myeloma cells (33).

HK1^HK2^ RPMI8226 multiple myeloma cells are sensitive to proliferation inhibition by the hHk2 ASO. However, expression of HK1 in the parental HK1^HK2^
RPMI8226 multiple myeloma cells rendered the resulting RPMI8226 isogenic HK1⁺HK2⁺ cells resistant to the inhibitory effect of hHK2 ASO on cell proliferation. In animal xenografts bearing human multiple myeloma tumors the hHK2 ASOs, dissolved in PBS and injected subcutaneously, were able to dramatically reduce tumor hHK2 protein levels (33).

Because hHK2 ASOs do not inhibit mouse HK2 (33), HK2 ASOs specific for mouse HK2 (mHK2 ASOs) were also developed, to examine their tolerability by normal tissues. mHK2 ASOs had no adverse effects when administered to allogeneic mouse models with mouse multiple myeloma HK1⁻HK2⁺ cells, validating the safety of ASO HK2 knockdown. These data suggest the likely safety of selective HK2 inhibitors for clinical use following systemic administration.

**Synthetic lethality combination therapy with HK2 inhibition for treating HK1⁺HK2⁺ cancers.**

Although effective in slowing HK1⁺HK2⁺ cancer cell proliferation, HK2 inhibition alone is cytostatic, not cytotoxic, in culture. In addition, HK2 inhibition slows, but does not eliminate, HK1⁺HK2⁺ cancer cell progression in xenograft tumors (33,34,37). In cultured HK1⁺HK2⁺ liver cancer cells, when HK2 was allowed to re-express by removing the shRNA HK2 suppression, cells were able to regrow (37). In xenograft models, shHK2 knockdown reduced growth rates of HK1⁺HK2⁺ Hep3B liver tumors and isogenic H460/HK1⁺HK2⁺ lung tumors by approximately 50% (34). These observations
suggested that other energy production pathways may compensate for the reduction of glycolysis by HK2 inhibition and maintain cancer cell survival.

To systematically identify co-dependent pathways and develop a synthetic lethality therapy to treat HK1-HK2+ cancers, a high throughput screen of drug-like compounds, compounds in clinical trials, and approved oncology drugs was performed (37). The potent mitochondrial complex I inhibitor diphenyleneiodonium (DPI) was identified as the best synthetic lethality partner for cultured HK1-HK2+ Hep3B liver cancer cells when combined with shHK2 knockdown (Fig 2A) (37).

Although HK2 also plays an important role in mediating NADPH regeneration through the oxidative branch of the pentose phosphate pathway (PPP), shRNA knockdown of glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting and NADPH regenerating enzyme in the oxidative branch of PPP (7), did not show synthetic lethality with shHK2 inhibition in HK1-HK2+ liver cancer Hep3B cells (Fig. 2A), in contrast to our results observed for the interaction between shHK2 and DPI. These results suggest that the synthetic lethality of the combination of mitochondrial complex I inhibition by DPI and glycolysis inhibition by shHK2 is not mediated by NADPH insufficiency.

In xenograft models, DPI and shHK2 synergistically suppressed HK1-HK2+ liver tumor growth (Fig. 2B) and induced tumor cell apoptosis (37). Inhibition of glycolysis has been documented to induce co-dependency on other energy production pathways (37,43); oxidative phosphorylation (OXPHOS) in particular (44). Glycolysis and OXPHOS pathways are linked by a core circuit containing HIF-1, AMPK, and ROS, as well as oncogenes such as RAS, MYC, and c-SRC (45-47). Multiple studies using
various models have supported that HK inhibition by 2-deoxy-D-glucose (2DG), a derivative of glucose and an HK1 and HK2 inhibitor, causes an increase in the OXPHOS level and results in a synergy between 2DG and OXPHOS inhibitors (48-50).

The co-dependency on OXPHOS when glycolysis is inhibited in HK1^−HK2^+ cancer cells is caused by two effects of this inhibition on cellular ATP reduction. First, glycolysis generates two ATP molecules from each glucose molecule consumed; glycolysis inhibition results in direct reduction in ATP production. In addition, inhibition of glycolysis decreases the production and consequent intracellular levels of pyruvate, an acetyl-CoA precursor which enters the tricarboxylic acid cycle to ultimately produce ATP through OXPHOS. As a result, ATP loss due to glycolysis inhibition requires compensating ATP production through OXPHOS by the use of other fuel sources such as amino acids or fatty acids.

When both HK2-mediated glycolysis and OXPHOS pathways were reduced in HK1^−HK2^+ cancer cells by the shHK2 and DPI combination, fatty acid oxidation (FAO) was up-regulated to support cancer cell survival (37). Inhibition of FAO by carnitine palmitoyltransferase I (CPT1) inhibitors, including perhexiline (PER, approved as an anti-anginal drug in Australia, New Zealand, and Asia, and in clinical trials in the U.S.) and etomoxir (a research tool), sensitized HK1^−HK2^+ cancer cells to the combination of HK2 inhibition and OXPHOS reduction. The triple combination of shHK2 knockdown, DPI OXPHOS reduction, and PER FAO inhibition kills both naturally existing HK1^−HK2^+ cancer cells and isogenic HK1^KO^HK2^+ cancer cells in culture. This triple combination also suppressed HK1^−HK2^+ tumor progression in xenograft models (Fig. 2C) (37). Moreover, no adverse effects were observed in the xenograft animals. In contrast, this
combination therapy, effective for HK1\(^+\)HK2\(^+\) cancers, is tolerated both by HK1\(^+\)HK2\(^+\) cancer cells in culture and HK1\(^+\)HK2\(^+\) tumor xenografts (Figs. 2D and E) (33,37). These data demonstrate that the combination of HK2 inhibition, along with partial inhibition of OXPHOS and FAO, is a promising therapeutic strategy for treating HK1\(^+\)HK2\(^+\) cancers.

While DPI is a potent inhibitor of mitochondrial complex I, it has not been tested in human subjects. Metformin (MET), a clinically approved drug, is also an inhibitor of mitochondrial complex I. To translate this triple component therapeutic strategy towards clinical treatment for HK1\(^+\)HK2\(^+\) cancers, an hHK2 ASO (hHK2-ASO1) was combined with MET and PER. This hHK2-ASO/MET/PER combination suppressed the growth of both subcutaneous and disseminated xenograft models of human HK1\(^+\)HK2\(^+\) multiple myeloma (Fig. 2F and G) (33).

Both DPI and MET have known off-target effects. For example, DPI has been reported to also inhibit NADPH oxidase (NOX) and nitric oxide synthase (NOS) (37). We demonstrated that neither NOX inhibitors (GKT137831 and apocynin) nor NOS inhibitors (L-NAME and L-NNA) showed synergy with HK2 blockade in HK1\(^+\)HK2\(^+\) cancers (37). On the other hand, we confirmed that other small molecule inhibitors of mitochondrial complex I, such as rotenone (37), also showed synergy with HK2 blockade in HK1\(^+\)HK2\(^+\) cancers (37).

Because of sequence differences for human and mouse HK2 mRNAs, HK2 expression in normal mouse tissues is not sensitive to hHK2-ASOs tested in xenograft models. To further evaluate the safety of the triple combination therapy, the mouse HK2 ASO (mHK2-ASO1) described above was tested in combination with MET and PER in mouse tumor progression models with both subcutaneous and disseminated
mouse HK1-HK2+ multiple myeloma. In both models, the triple combination suppressed tumor progression and was well tolerated (Fig. 2H and I) (33).

As determined by mass spectrometry, the triple combination synergistically inhibited energy (ATP) production in HK1-HK2+ cancer cells (33,37), as expected from our viability studies. The depletion of other metabolites may also contribute to the synthetic lethality; a suggestion that needs further investigation. In addition, future studies will be needed to evaluate the efficacy of the combination of HK2 ASO, MET and PER in HK1-HK2+ cancer cells from other cancer types.

Conclusions and future directions

Ever since the Warburg effect was first reported (1), inhibition of glycolysis has been a proposed and sought-after target for cancer therapy. However, retrospectively, we now understand that inhibition of tumor glycolysis by suppressing global hexokinase activity has proved to be an elusive cancer therapeutic target because of the commonality of HK1, both to many tumors and to most normal tissues, and the consequent adverse effects of global HK inhibition. With the discovery that HK1 expression in most normal tissues is likely to be responsible for the adverse effects of attempts at therapy through global inhibition of HK activity, and the observation that ectopic HK2 expression occurs in most cancers, the hypothesis that selective HK2 inhibition would provide therapeutic benefit became popular. However, we now understand that even if HK2 is selectively inhibited in tumors, with consequent reduced
adverse effects, the HK1 present in many tumors appears sufficient to sustain tumor progression.

However, all is not lost! Subsets of HK1^HK2^ cancers exist, in varying percentages in cancers, regardless of their tissues of origin. While the efficacy of selective HK2 inhibitors is likely to be limited to HK1^HK2^ cancers, it appears that inhibition of glycolysis as an effective therapeutic contribution to this subset of cancers is on the verge of effective clinical translation. The combination of specific HK2 inhibition, partial OXPHOS inhibition, and partial FAO inhibition is a promising combination therapy for HK1^HK2^ cancers, regardless of tissues of origin, and would likely be well tolerated by normal tissues. As a result of these studies, we anticipate that searches for small molecule HK2 selective inhibitors will once again gain favor. Future studies to define the threshold levels of HK1 that can support tumor sustaining glycolysis activity in individual cancer types when HK2 is inhibited would help extend this combination therapy to HK2^ cancers with HK1 expression below such thresholds.

The NCI Dictionary of Cancer Terms defines “precision medicine” as using “specific information about a person’s tumor to help diagnose, plan treatment, find out how well treatment is working or make a prognosis.” We consider the use of targeted HK2 inhibition restricted to tumors identified as HK1^HK2^ to meet with the definition of an application of the principle of “precision medicine”. The NCI Dictionary of Cancer Terms defines “tumor agnostic therapy” as “a type of therapy that uses drugs or other substances to treat cancer based on the cancer’s genetic and molecular features, without regard to the cancer type or where the cancer started in the body.” We would conclude, from this definition, that HK2 targeted selective therapy would also be
regarded as a tumor agnostic therapy for cancers that are HK1\(^{-}\)HK2\(^{+}\) regardless of tissue of origin. The basic conclusions for agnostic therapy of HK1\(^{-}\)HK2\(^{+}\)-tumors by inhibition of glycolysis, oxidative phosphorylation and fatty acid oxidation are summarized in Fig. 2J.

**Acknowledgements**

We thank our many colleagues who participated in the design and execution of experiments described in this review. We particularly acknowledge the participation of Drs. Sherie Morrison, Sarah Larson and John Timmerman in studies on multiple myeloma, and Drs. Tianyuan Zhou, Rob MacLeod and Youngsoo Kim for their design, production and characterization of the hHK2 and mHK2 ASOs. We thank Drs. Sherie Morrison, Tomas Graeber, and David Nathanson for critical reading of the manuscript. HRH was supported by the Crump Family Foundation and the Phelps Family Foundation. SX was a FY15 Horizon Awardee (W81XWH-16-1-0162) supported by the DoD Peer Reviewed Cancer Research Program.
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Figure Legends:

Figure 1. HK2 is redundant for tumor progression and does not contribute a selective advantage in HK1\(^+\)HK2\(^+\) cancers, but is essential for optimal HK1\(^+\)HK2\(^+\) cancer progression. (A) Scheme of experimental procedure to demonstrate that HK2 does not provide a selective advantage to tumor progression in HK1\(^+\)HK2\(^+\) tumors. 0.5×10\(^5\) isogenic H460 HK1\(^+\)HK2\(^+\) and 4.5×10\(^5\) HK1\(^+\)HK2\(^-\) cells were mixed before subcutaneous injection into six nu/nu mice. The residual cell mixture left from injection was lysed and frozen. During tumor growth, three tumors were harvested at an early time point when the tumor size was relatively small. The other three tumors were collected at a late time point when the tumor size was relatively large. HK1 and HK2 protein levels were compared among the sample remaining from injection, the early tumors, and the late tumors derived from the isogenic H460 HK1\(^+\)HK2\(^+\) and HK1\(^+\)HK2\(^-\) cell mixtures, using Western blotting. (B) Tumor growth and weight of mixed early/small tumors and late/large tumors. Mixed tumors were collected at an early/small stage and at a late/large stage, as shown. (C) Immunoblot analysis of HK1 and HK2 protein levels in mixed early/small tumors and late/large tumors, compared to the tumor cell mixture used for subcutaneous injection. A “standard curve” of cell lysates of isogenic HK1\(^+\)HK2\(^+\) and HK1\(^+\)HK2\(^-\) H460 cells at the mixed ratios indicated is also presented. (D) doxycycline (DOX)-induced shHK2 knockdown does not affect the progression of parental H460/ HK1\(^+\)HK2\(^+\) subcutaneous xenograft tumors. (E) DOX-induced shHK2 knockdown significantly reduces the progression of isogeneic H460/ HK1\(^+\)HK2\(^+\) subcutaneous xenograft tumors. * P<0.05. ns, not significant. Panels D and E are reprinted from Xu et al (34).
Figure 2. Co-inhibition of HK2, OXPHOS, and FAO is a promising therapeutic strategy for HK1\(^{-}\)HK2\(^{+}\) cancers. (A) The combination of DPI and shHK2 causes synthetic lethality in HK1\(^{-}\)HK2\(^{+}\) Hep3B cells. In contrast, the combination of DPI/shG6PD does not affect viability of these cells. Hep3B/shHK2\(^{DOX}\) and Hep3B/shG6PD\(^{DOX}\) cells were pretreated with doxycycline (DOX) for 48 h, prior to the treatments with DOX and/or DPI (100 nM) for 72 h. Left panel, time course effects of DOX-mediated HK2 knockdown and G6PD knockdown were determined by Western blotting. Right panel, cell viability was determined by trypan blue staining. (B) DOX-induced shHK2 and DPI synergistically suppress subcutaneous (s.c.) liver cancer Hep3B (HK1\(^{-}\)HK2\(^{+}\)) xenograft tumor progression. (C) Perhexiline (PER) sensitizes Hep3B (HK1\(^{-}\)HK2\(^{+}\)) xenograft tumors to the shHK2/DPI combination inhibition of tumor progression. (D) The combination of shHK2, DPI and PER suppresses isogenic H460/HK1\(^{-}\)HK2\(^{+}\) xenograft tumor progression, but has no significant effect on parental H460/HK1\(^{-}\)HK2\(^{+}\) tumor progression. (E) The combination of hHK2-ASO1, DPI and PER has no significant effect on progression of s.c. U266/HK1\(^{-}\)HK2\(^{+}\) multiple myeloma xenograft tumors. (F) The combination of hHK2-ASO1, DPI (or MET) and PER suppresses progression of s.c. OPM2/HK1\(^{-}\)HK2\(^{+}\) multiple myeloma xenograft tumors. (G) The combination of hHK2-ASO1, MET and PER suppressed the progression of disseminated RPMI8226/HK1\(^{-}\)HK2\(^{+}\) multiple myeloma xenografts. RPMI8226/HK1\(^{-}\)HK2\(^{+}\) multiple myeloma cells stably express firefly luciferase (fLUC); tumor progression was monitored by bioluminescence imaging (BLI). (H) The combination of mHK2-ASO1, MET and PER suppresses progression of s.c. mouse P3/HK1\(^{-}\)HK2\(^{+}\) multiple myeloma tumors. (I) The combination
of mHK2-ASO1, MET and PER suppressed the progression of disseminated mouse P3/HK1−HK2+ multiple myeloma tumors. (J) A graphical summary of the tumor agnostic therapeutic strategy for HK1−HK2+ cancers. * P<0.05. ** P<0.01. *** P<0.001. **** P<0.0001. ns, not significant. Panels B-D and the HK2 portion in panel A are reprinted from Xu et al (37); Panels E-I are reprinted from Xu et al (33).
HK1+HK2+ H460 cells

HK1+HK2– H460 cells

10%
90%

Early/small
Late/large

H460/HK1+HK2+/shHK2DOX xenografts:

H460/HK1–HK2+/shHK2DOX xenografts:

HK1

HK2

GAPDH

HK1

HK2

GAPDH

Tumor volume (mm³)

Time after implantation (d)

Pre-inoculation
0 5 10 15
Early/small tumors:

Late/large tumors:

Tumor volume (mm³)

Time on treatment (d)

Pre-inoculation
0 5 10 15

Early/small tumors:

Late/large tumors:

H460/HK1+HK2+/shHK2DOx xenografts:

H460/HK1–HK2+/shHK2DOx xenografts:

D

E

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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A tumor agnostic therapeutic strategy for HK1–HK2+ cancers

**A**
Hep3B/HK1–HK2+/shHK2DOX s.c. xenografts:

**B**
Hep3B/HK1–HK2+/shHK2DOX s.c. xenografts:

**C**
Hep3B/HK1–HK2+/shHK2DOX s.c. xenografts:

**D**
H460/HK1+HK2+/shHK2DOX s.c. xenografts:

**E**
U266/HK1–HK2+/shHK2DOX s.c. xenografts:

**F**
OPM2/HK1+HK2+/shHK2DOX s.c. xenografts:

**G**
RPMI8226/HK1–HK2+ disseminated xenografts:

**H**
P3/HK1+HK2+/shHK2DOX s.c. allogeneic grafts:

**I**
P3/HK1+HK2+ disseminated allogeneic grafts:

**J**
A tumor agnostic therapeutic strategy for HK1–HK2+ cancers
A tumor agnostic therapeutic strategy for Hexokinase 1 Null/Hexokinase 2 positive cancers

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Cancer Res  Published OnlineFirst August 21, 2019.

Updated version

Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-19-1789

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