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# A lymphoblast model for IDH2 gain-of-function activity in D-2-hydroxyglutaric aciduria type II: Novel avenues for biochemical and therapeutic studies

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ABSTRACT

The recent discovery of heterozygous isocitrate dehydrogenase 2 (IDH2) mutations of residue $Arg^{140}$ to $Gln^{140}$ or $Gly^{140}$ ($IDH2^{wt/R140Q}$, $IDH2^{wt/R140G}$) in D-2-hydroxyglutaric aciduria (D-2-HGA) has defined the primary genetic lesion in 50% of D-2-HGA patients, denoted type II. Overexpression studies with $IDH1^{R132H}$ and $IDH2^{R172K}$ mutations demonstrated that the enzymes acquired a new function, converting 2-ketoglutarate (2-KG) to D-2-hydroxyglutarate (D-2-HG), in lieu of the normal IDH reaction which reversibly converts isocitrate to 2-KG. To confirm the $IDH2^{wt/R140Q}$ gain-of-function in D-2-HGA type II, and to evaluate potential therapeutic strategies, we developed a specific and sensitive $IDH2^{wt/R140Q}$ enzyme assay in lymphoblasts. This assay determines gain-of-function activity which converts 2-KG to D-2-HG in homogenates of D-2-HGA type II lymphoblasts, and uses stable-isotope-labeled 2-keto[3,3,4,4-$^2H_4$]glutarate. The specificity and sensitivity of the assay are enhanced with chiral separation and detection of stable-isotope-labeled D-2-HG by ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Eleven potential inhibitors of $IDH2^{wt/R140Q}$ enzyme activity were evaluated with this procedure. The mean reaction rate in D-2-HGA type II lymphoblasts was 8-fold higher than that of controls and D-2-HGA type I cells (14.4 nmol $h^{-1}$ mg protein $^{-1}$ vs. 1.9), with a corresponding 140-fold increase in intracellular D-2-HG level. Optimal inhibition of $IDH2^{wt/R140Q}$ activity was obtained with oxaloacetate, which competitively inhibited $IDH2^{wt/R140Q}$ activity. Lymphoblast $IDH2^{wt/R140Q}$ showed long-term cell culture stability without loss of the heterozygous $IDH2^{wt/R140Q}$ mutation, underscoring the utility of the lymphoblast model for future biochemical and therapeutic studies.



## 1. Introduction

The discovery of recurrent mutations in isocitrate dehydrogenase 1 (IDH1) in patients with glioblastoma multiforme has rekindled interest in NADP(H)-dependent isocitrate dehydrogenase enzymes [1]. A high incidence of specific mutations in the active sites of cytosolic and peroxisomal IDH1 (OMIM ID: 147700) (residue $Arg^{132}$/R132) and the mitochondrial homologue IDH2 (OMIM ID: 147650) (residues $Arg^{140}$/R140 and $Arg^{172}$/R172) are found in a wide variety of hematologic malignancies and solid tumors [2]. These mutations alter the enzymes normal capacity to convert isocitrate to 2-ketoglutarate (2-KG) and confer on it a new function which converts 2-KG to D-2-hydroxyglutarate (D-2-HG), leading to accumulation of D-2-HG in solid tumor cells and in plasma of patients with acute myeloid leukemia [3,4].

Supraphysiological D-2-HG concentrations in body fluids are the biochemical hallmark of the inborn errors of metabolism D-2-hydroxyglutaric aciduria (D-2-HGA) type I (OMIM ID: 600721) and type II (OMIM ID: 613657). D-2-HGA type I is caused by various homozygous/compound heterozygous mutations/deletions in the *D2HGDH* gene encoding for D-2-hydroxyglutarate dehydrogenase (D-2-HGDH, OMIM ID: 609186) [5], whereas D-2-HGA type II is caused primarily by the heterozygous *de novo* $IDH2^{wt/R140Q}$ mutation [6]. Thus far only one patient was detected with an $IDH2^{wt/R140G}$ mutation. The phenotype of D-2-HGA has a broad clinical spectrum ranging from asymptomatic to a severe clinical presentation with heterogeneous MRI abnormalities observed [7–10]. Another form of D-2-HGA is associated with spondyloenchondromatosis, but the etiology is unknown [11]. The chiral

*Abbreviations:* 2-KG, 2-ketoglutarate; $^2H_4$-2-KG, 2-keto[3,3,4,4-$^2H_4$]glutarate; $^2H_4$-D-2-HG, D-2-[3,3,4,4-$^2H_4$]hydroxyglutarate; D-2-HG, D-2-hydroxyglutarate; D-2-HGA, D-2-hydroxyglutaric aciduria; D-2-HGDH, D-2-hydroxyglutarate dehydrogenase; Datan, (+)-O,O′-Diacetyl-L-tartaric anhydride; HOT, hydroxyacid-oxoacid transhydrogenase; IDH, isocitrate dehydrogenase; $IDH1^{wt/wt}$, isocitrate dehydrogenase 1 wild type; $IDH2^{wt/R140Q}$, heterozygous isocitrate dehydrogenase 2 mutation of residue $Arg^{140}$ to $Gln^{140}$; L-2-HG, L-2-hydroxyglutarate; L-2-HGA, L-2-hydroxyglutaric aciduria; L-2-HGDH, L-2-hydroxyglutarate dehydrogenase; UPLC-MS/MS, ultra performance liquid chromatography-tandem mass spectrometry

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counterpart of D-2-HG, L-2-hydroxyglutarate (L-2-HG), is increased in patients with L-2-hydroxyglutaric aciduria (L-2-HGA, OMIM ID: 236792), a leukoencephalopathy associated with mutations in the *L2HGDH* gene encoding for L-2-hydroxyglutarate dehydrogenase (L-2-HGDH, OMIM ID: 609584) [12–14]. Whereas an increased incidence of brain tumors is reported in L-2-HGA [15], currently no neoplastic malignancies have been reported in D-2-HGA.

A specific enzyme assay using enantiomerically pure stable-isotope-labeled D-2-HG was developed to assess the enzyme activity of D-2-HGDH in cultured fibroblasts and lymphoblasts, verifying that D-2-HGDH was impaired in D-2-HGA type I patients [16]. An enzyme assay to assess $IDH2^{wt/R140Q}$-mutant enzyme activity in cultured human cells from D-2-HGA type II patients has not been described. Several groups used spectrophotometric assays to study $IDH1^{R132H}$, $IDH1^{R132C}$, $IDH2^{R140Q}$, $IDH2^{R140W}$, and $IDH2^{R172K}$ enzyme activities via the NADP(H) couple in overexpressed cells [3,4,17], yet this approach suffered from high background issues in extracts of cultured human cells. Pietrak et al. improved the assay's characteristics by employing tandem mass spectrometry to quantify 2-hydroxyglutarate, however without separation of D- and L-2-HG analytes [18]. We sought to develop a more robust assay system which could both verify the presence of the $IDH2^{wt/R140Q}$ gain-of-function activity and simultaneously investigate potential therapeutic inhibitors which might quantitatively decrease D-2-HG production. Accordingly, we developed a new assay which determines $IDH2^{wt/R140Q}$ activity in lymphoblast extracts using stable-isotope-labeled 2-keto[3,3,4,4-$^2H_4$]glutarate ($^2H_4$-2-KG) as substrate. The reaction product, D-2-[3,3,4,4-$^2H_4$]hydroxyglutarate ($^2H_4$-D-2-HG), is specifically detected by derivatization and chiral separation of D-2-HG and L-2-HG using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Further, this new assay facilitated the preclinical evaluation of several endogenous metabolites for their ability to inhibit $IDH2^{wt/R140Q}$ activity.

## 2. Materials and methods

### 2.1. Lymphoblast cell lines and protein extraction

All lymphoblast cell lines were obtained from Epstein–Barr virus transfected lymphocytes and were anonymized. Five cell lines were obtained from D-2-HGA type II patients which all carried the heterozygous *IDH2* c.[419 G>A], p.[Arg140Gln] ($IDH2^{wt/R140Q}$) mutation and cell culture stability was evaluated by confirmation of the presence of the heterozygous $IDH2^{wt/R140Q}$ mutation by DNA sequencing analysis. Additionally, five cell lines obtained from healthy individuals served as controls, supplemented with two cell lines obtained from D-2-HGA type I patients which carried compound heterozygous mutations in *D2HGDH* associated with impaired D-2-HGDH enzyme activity [5].

Lymphoblasts were grown in RPMI 1640 culture medium supplemented with 1% penicillin/streptomycin and 8% fetal bovine serum (all from Invitrogen) until a sufficient cell suspension was achieved to obtain a cell pellet containing ~$10^6$ cells. Subsequently, cells were harvested by centrifugation (6 min 340 g), washed twice with Hank's balanced salt solution and stored as a dry cell pellet at −80 °C prior to analysis.

Cell pellets were suspended in 250 μL M-PER containing 1% HALT protease/phosphatase inhibitor (Thermo Scientific), sonicated using a Bandalin Sonopuls mini 20 titanium 1.5 mm probe for 10 s at 90% power while cooled on ice, followed by centrifugation of the cell extract for 15 min at 14,000 g. The clarified supernatant was transferred to a 1.5 mL Eppendorf tube and gently mixed. Protein concentration was determined with a Pierce BCA protein assay (Sigma-Aldrich).

### 2.2. Incubation and UPLC-MS/MS analysis

The assay was performed in duplicate for 30 min at 37 °C in 150 μL buffer pH = 7.5 containing 100 mM Tris–HCl, 5 mM $MnCl_2$, 0.2 mM NADPH (Sigma-Aldrich), 15 mM 2-keto[3,3,4,4-$^2H_4$]glutarate ($^2H_4$-2-KG) (Euriso-Top, Gif sur Yvette, France) with a final protein concentration of 0.40 mg $mL^{-1}$. The reaction was terminated by cooling the samples on ice directly followed by protein removal via centrifugation with Microcon Ultracel YM-10 kDa filters (Millipore) in a pre-chilled centrifuge (15 min 20,000 g 4 °C). Twenty microliters of filtrate was transferred to a 1.5 mL HPLC vial, mixed with 200 pmol D/L-2-hydroxy[2-$^2H_1$]glutarate as internal standard in 200 μL methanol, and dried with $N_{2(g)}$ at 40 °C ($^2H_1$-D/L-2-HG, made in-house from unlabeled 2-KG by chemical reduction with zinc in deuterated water [24]). Subsequently, 50 μL (+)-O,O′-Diacetyl-L-tartaric anhydride (Datan, Aldrich) derivative reagent (50 mg $mL^{-1}$ dissolved in dichloromethane:acetic acid 80:20 (v/v)) was added, and the vials were capped and heated 30 min 75 °C. Finally, the samples were dried with $N_{2(g)}$ at 40 °C and dissolved in 500 μL mobile phase A (details below). Calibration lines of 10 to 400 pmol for each metabolite were composed of unlabeled D/L-2-HG (Sigma) and $^2H_4$-D/L-2-HG (made in-house from $^2H_4$-2-KG [24]). The substrate $^2H_4$-2-KG is converted by $IDH2^{wt/R140Q}$ into $^2H_4$-D-2-HG (Fig. 1), and the latter is determined by derivatization with Datan, followed by UPLC-MS/MS analysis [19], as a measure of the enzyme activity. Since the method allows the separation of D- and L-2-hydroxyglutarate, account was taken of the fact that D-2-hydroxyglutarate amounts to half of the total amount of 2-hydroxyglutarate in the standards.

For chromatographic analysis, a Waters Acquity UPLC was equipped with an HSS T3 column (2.1 × 100 mm 1.8 μm particle size) running at 0.4 mL $min^{-1}$ 97% mobile phase A (120 mg $mL^{-1}$ ammonium formate pH = 3.25) and 3% acetonitrile followed by a 40% acetonitrile wash step. Injection volume was 5 μL and detection was performed with an Applied Biosystems 4000 Q Trap mass spectrometer equipped with an ESI Turbo Spray interface running in negative MRM mode: transitions (m/z) D/L-2-HG = 363.0>146.9; $^2H_1$-D/L-2-HG = 364.0>147.9; $^2H_4$-D/L-2-HG = 367.0>150.9. Other settings included: CUR = 10; TEM = 100; GS1 = 40; GS2 = 50; ihe = ON; IS = −4500; CAD = 3; DP = −20; EP = −5; CE = −12.5; CXP = −10. The retention times were 2.8 min for L-2-HG and 3.4 min for D-2-HG (Fig. S1). Raw data processing was performed using Analyst 1.4.2./MS Excel and concentrations were extrapolated from calibration lines (Fig. S2).

### 2.3. $IDH2^{wt/R140Q}$ inhibition

Eleven (endogenous) metabolites were selected based upon their structural similarities with 2-KG, D-2-HG or isocitrate to test their capacity to reduce $^2H_4$-D-2-HG production by $IDH2^{wt/R140Q}$: D-2-HG, L-2-HG, L-isocitrate ($D_s$-(+)-*threo*-isocitric acid monopotassium salt), citrate, L-glutamate, L-glutamine, L-malate, D-malate, oxaloacetate, acetoacetate and acetoacetic acid methyl ester. Inhibitors were evaluated at up to ~30 mM concentration. All other parameters were held constant, as was the $^2H_4$-2-KG substrate concentration of 15 mM. The pH of the reaction mixture was monitored before and after incubation to verify constant experimental conditions, especially important since $IDH2^{wt/R140Q}$ is sensitive to small pH changes. The $K_i$ of oxaloacetate was evaluated by determining the $K_M$ of $^2H_4$-2-KG with 0.0, 5.8 and 11.7 mM oxaloacetate supplemented in the reaction mixture.

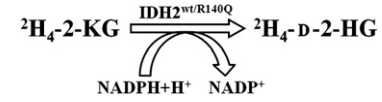


**Fig. 1.** Reaction mechanism catalyzed by $IDH2^{wt/R140Q}$ converting stable isotope labeled $^2H_4$-2-KG into $^2H_4$-D-2-HG with NADPH as electron donor.

## 3. Results

### 3.1. IDH2$^{wt/R140Q}$ gain-of-function enzyme assay

Optimal pH and concentrations of assay components (TRIS, $MnCl_2$, NADPH) were determined under conditions of the highest reaction rates of IDH2$^{wt/R140Q}$ in cell lysates of D-2-HGA type II lymphoblasts (data not shown). The reaction rate was stable over 60 min and the reaction dependence on protein content was linear to 1.14 mg protein mL$^{-1}$ (data not shown). Substrate dependence for IDH2$^{wt/R140Q}$ was determined in D-2-HGA type II cells with $K_{M,\ 2\text{-}KG} = 1.7$ mM and $V_{max} = 13.9$ nmol h$^{-1}$ mg protein$^{-1}$. The observed $K_{M,\ 2\text{-}KG}$ is of the same magnitude as previously reported for IDH1$^{R132H}$ overexpressed in cells: $K_{M,\ 2\text{-}KG} = 0.965$ mM [3] and $K_{M,\ 2\text{-}KG} = 0.7$ mM [18], whereas no $K_{M,\ 2\text{-}KG}$ has been previously reported for IDH2-mutants. $^2H_4$-D-2-HG production was also detected in control cells as well, produced by an unidentified enzyme, which had different characteristics of substrate dependence as compared to IDH2$^{wt/R140Q}$: $K_{M,\ 2\text{-}KG} = 0.7$ mM and $V_{max} = 2.0$ nmol h$^{-1}$ mg protein$^{-1}$ (Fig. 2).

The mean reaction rate detected in control (n = 5) and D-2-HGA type I (n = 2) lymphoblasts was 1.8 and 1.9 nmol h$^{-1}$ mg protein$^{-1}$ respectively, whereas in D-2-HGA type II (n = 5) the mean reaction rate was ~8 times higher: 14.4 nmol h$^{-1}$ mg protein$^{-1}$ (Fig. 3). This resulted in increased intracellular D-2-HG concentrations in D-2-HGA type II cells when compared to controls/D-2-HGA type I cells. The mean endogenous D-2-HG (nmol mg protein$^{-1}$) values were: controls = 0.11, D-2-HGA type I = 1.80, and D-2-HGA type II = 15.06 (Fig. 4). The higher concentration of D-2-HG in D-2-HGA type II compared to type I is consistent with the trend observed in body fluids as previously reported [5,6]. Conversely, the intracellular L-2-HG concentrations were comparable among all twelve cell lines, verifying the independence of this metabolite from D-2-HG (Fig. 5). Furthermore, small amounts of $^2H_4$-L-2-HG were detected, which were also equivalent between all cell lines. $^2H_4$-L-2-HG most likely originates from the nonspecific conversion of $^2H_4$-2-KG via L-malate dehydrogenase, an NADH-dependent reaction [20]. In support of this observation, when NADPH was replaced by NADH in the assay buffer with D-2-HGA type II cells, $^2H_4$-L-2-HG production increased 24-fold, whereas $^2H_4$-D-2-HG production decreased 9-fold (data not shown). The IDH2$^{wt/R140Q}$ assay demonstrated little diagnostic sensitivity in fibroblast homogenates in its ability to distinguish between IDH2$^{wt/R140Q}$ enzyme activity and that observed in controls. The $^2H_4$-D-2-HG production in D-2-HGA type II lysates was only twice that observed in controls/D-2-HGA type I cells, which accounted for the observed intracellular D-2-HG accumulation as well (data not shown).

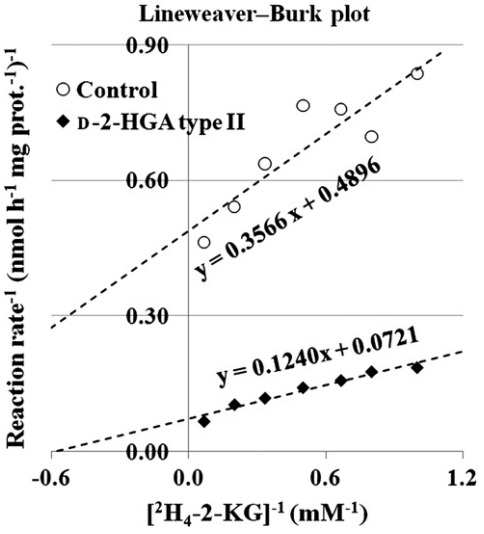


**Fig. 2.** Lineweaver-Burk plot showing the reciprocal reaction rate as a function of reciprocal $^2H_4$-2-KG concentration for: (♦) IDH2$^{wt/R140Q}$ activity in D-2-HGA type II cell homogenate [$K_M = 1.7$ mM and $V_{max} = 13.9$ nmol h$^{-1}$ mg protein$^{-1}$] and (○) unidentified enzyme in control cell homogenate [$K_M = 0.7$ mM and $V_{max} = 2.0$ nmol h$^{-1}$ mg protein$^{-1}$].

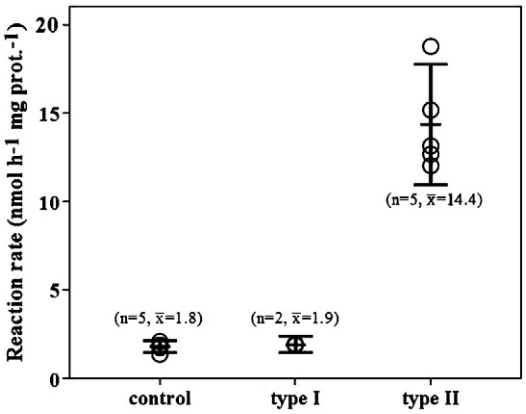


**Fig. 3.** Reaction rates (nmol h$^{-1}$ mg protein$^{-1}$) determined in cell homogenates of controls (n = 5, mean = 1.8), D-2-HGA type I (n = 2, mean = 1.9) and D-2-HGA type II cells (n = 5, mean = 14.4), error bars: 95% CI (SPSS 15.0).

### 3.2. IDH2$^{wt/R140Q}$ inhibition

From eleven metabolites used to screen for inhibition of IDH2$^{wt/R140Q}$, oxaloacetate was the most potent: the $^2H_4$-D-2-HG production was more than 50% reduced when equal concentrations (15 mM) of substrate and inhibitor were used (Fig. 6). Decreasing reaction rates and increasing $K_M$ for $^2H_4$-D-2-HG were observed when 2-KG substrate dependence was tested for IDH2$^{wt/R140Q}$ with varying oxaloacetate concentration and a $K_{i,\ oxaloacetate}$ of 3.9 mM was computed from the data (Fig. 6). Oxaloacetate functions as a competitive inhibitor in IDH2$^{wt/R140Q}$ with a slightly lower selectivity compared to 2-KG, since $K_{i,\ oxaloacetate}/K_{M,\ 2\text{-}KG} = 2$.

## 4. Discussion

Biochemical mechanisms for heterozygous IDH1 and IDH2 mutations have been recently summarized. IDH1 and IDH2 are NADP(H)-dependent homodimers catalyzing the reversible conversion of isocitrate to 2-KG. Zhao et al. observed that heterodimer formation between a wild type and mutant subunit (IDH1$^{wt/R132H}$) resulted in considerable loss of isocitrate to 2-KG catalytic activity, while

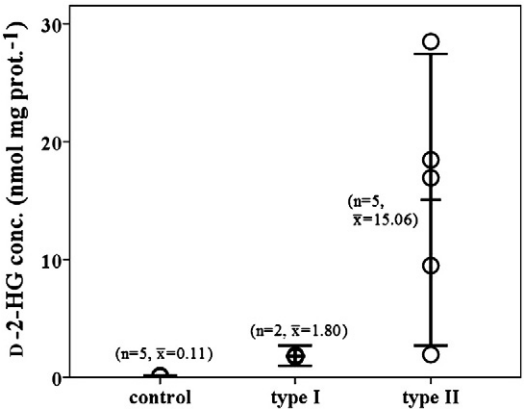


**Fig. 4.** Endogenous intracellular D-2-HG concentrations (nmol mg protein$^{-1}$) determined in cell homogenates of controls (n = 5, mean = 0.11), D-2-HGA type I (n = 2, mean = 1.80) and D-2-HGA type II (n = 5, mean = 15.06), error bars: 95% CI (SPSS 15.0).

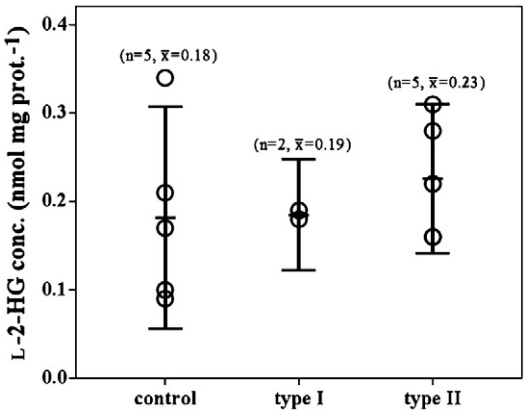


**Fig. 5.** Endogenous intracellular L-2-HG concentrations (nmol mg protein$^{-1}$) determined in cell homogenates of controls (n = 5, mean = 0.18), D-2-HGA type I (n = 2, mean = 0.19) and D-2-HGA type II (n = 5, mean = 0.23), error bars: 95% CI (SPSS 15.0).

the mutant IDH1$^{R132H/R132H}$ homodimer is completely devoid of this activity [21]. D-2-HG is formed from 2-KG and NADPH [3,4] in cells that overexpress IDH1$^{R132H}$ and IDH2$^{R172K}$. Pietrak et al. demonstrated that IDH1$^{wt/R132H}$ subunits function independently, resulting in a coupled reaction which produces 2-HG from isocitrate and NADP$^+$ [18], as had been previously hypothesized by Dang and Ward et al. Unfortunately, chiral differentiation of product 2-HG was not performed to differentiate the level of D- and L-2-HG production. In the coupled reaction sequence, isocitrate and NADP$^+$ are converted into 2-KG and NADPH via IDH1$^{wt}$-subunit catalysis, with subsequent production of 2-HG and NADP$^+$ via IDH1$^{R132H}$-subunit catalysis. Further, Pietrak and co-workers demonstrated production of 2-HG from 2-KG via IDH1 wild type (IDH1$^{wt/wt}$) catalysis, a reaction strongly inhibited by isocitrate. This reaction might represent an additional source of D-2-HG in humans in conjunction with the reaction catalyzed by hydroxyacid-oxoacid transhydrogenase (HOT, OMIM ID: 611083) [22]. HOT primarily converts gamma-hydroxybutyrate (GHB) to succinic semialdehyde coupled to the reduction of 2-KG to D-2-HG. However, HOT has specificity towards several other GHB-like metabolites which serve as electron donors as well. These reactions catalyzed by IDH$^{wt/wt}$ and HOT likely contribute to endogenous D-2-HG production observed in the IDH2$^{wt/R140Q}$ enzyme assay (Fig. 2), and most likely contributed a high proportion of the background activity in assays employing fibroblast extracts.

The concentration of D-2-HG is increased 140 fold in lymphoblasts from patients with IDH2$^{wt/R140Q}$-mutations compared to controls, whereas the enzymatic activity of D-2-HG production in cell free systems is increased by only 8-fold. Perhaps, the larger difference observed for intact cells is due to the fact that D-2-hydroxyglutarate dehydrogenase (D-2-HGDH) consumes essentially all the D-2-HG that is formed in control cells, but due to limited capacity [23], it is incapable of metabolizing all of the D-2-HG formed by IDH2$^{wt/R140Q}$. Accordingly, the amount of D-2-HG that accumulates in cells that are deficient in D-2-HGDH (and have a non-mutated IDH2) amounts to about 1/8th of that found in cells from patients with mutated IDH2. Additionally, our in vitro enzyme assay conditions may not exactly recapitulate those in the mitochondrial compartment, and thus the IDH2$^{wt/R140Q}$ reaction rate determined in vitro may be an underestimation of the endogenous intracellular rate.

The IDH2$^{wt/R140Q}$ assay was useful for exploring preclinical therapeutic interventions as well. Oxaloacetate acted as a competitive inhibitor for the conversion of 2-KG to D-2-HG. The comparable affinity of IDH2$^{wt/R140Q}$ for oxaloacetate and 2-KG likely relates to their structural similarity. The assay is also potentially useful to evaluate the capacity of mutated IDH1 or IDH2 to produce D-2-HG in extracts of cancerous cells and thereby take into account the amount of 'active' mutated IDH, which may vary among patients. Additionally, the assay can possible be employed to detect the effect of other, as yet undetected mutations, which might also convert IDH to a D-2-HG-producing enzyme.

In conclusion, the IDH2$^{wt/R140Q}$ gain-of-function enzyme assay we have developed is readily applicable to cultured lymphoblasts derived from D-2-HGA type II patients. Chiral separation and quantification of stable-isotope-labeled D-2-hydroxyglutarate by UPLC-MS/MS provided a highly specific and sensitive method, and an assay which facilitates rapid identification of D-2-HGA type II cells with IDH2$^{wt/R140Q}$ gain-of-function. Perhaps most important, heterozygous IDH2$^{wt/R140Q}$ mutations and gain-of-function activity are preserved intracellularly following long-term storage and cell culture, highlighting the utility of the lymphoblast model for future biochemical and therapeutic explorations.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbadis.2011.08.006.

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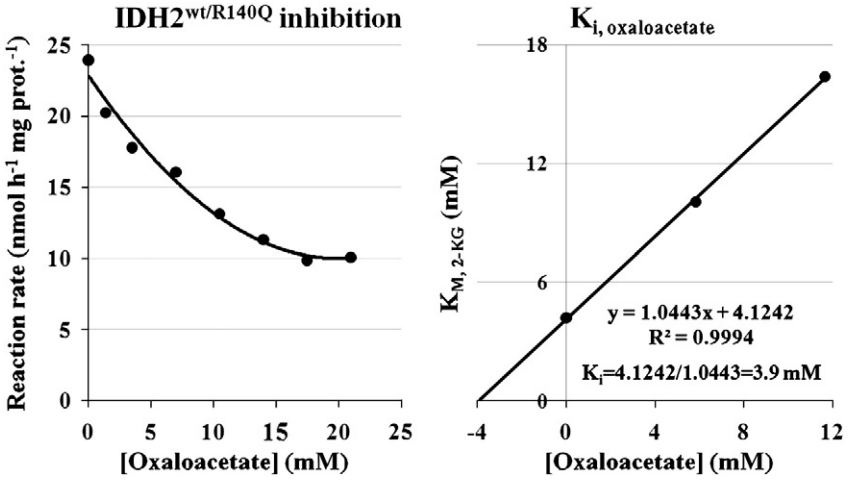


**Fig. 6.** [left] Inhibition of IDH2$^{wt/R140Q}$ reaction rate as a function of oxaloacetate concentration assessed in D-2-HGA type II cell homogenates using standard assay conditions ($^2H_4$-2-KG substrate conc. = 15 mM); [right] Observed $K_{M,\ 2\text{-}KG}$ with different oxaloacetate concentrations (0, 5.8 and 11.7 mM). The x-axis intercept of the regression line represents -$K_i$: $K_{i,\ oxaloacetate}$ = 3.9 mM.

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