

Identification of Novel Small-Molecule Inhibitors for Human Transketolase by High-Throughput Screening with Fluorescent Intensity (FLINT) Assay

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The metabolic enzyme transketolase (TK) plays a crucial role in tumor cell nucleic acid synthesis, using glucose through the elevated nonoxidative pentose phosphate pathway (PPP). Identification of inhibitors specifically targeting TK and preventing the nonoxidative PPP from generating the RNA ribose precursor, ribose-5-phosphate, provides a novel approach for developing effective anticancer therapeutic agents. The full-length human transketolase gene was cloned and expressed in *Escherichia coli* and the recombinant human transketolase protein purified to homogeneity. A fluorescent intensity (FLINT) assay was developed and optimized. Library compounds were screened in a high-throughput screening (HTS) campaign using the FLINT assay. Fifty-four initial hits were identified. Among them, 2 scaffolds with high selectivity, ideal physicochemical properties, and low molecular weight were selected for lead optimization studies. These compounds specifically inhibited in vitro TK enzyme activity and suppressed tumor cell proliferation in at least 3 cancer cell lines: SW620, LS174T, and MIA PaCa-2. Identification of these active scaffolds represents a good starting point for development of drugs specifically targeting TK and the nonoxidative PPP for cancer therapy. (*Journal of Biomolecular Screening* 2004:427-433)

Key words: transketolase, nonoxidative PPP, FLINT assay, HTS, small-molecule inhibitors

INTRODUCTION

TRANSKETOLASE (TK; D-sedoheptulose-7-phosphate: D-glyceraldehyde-3-phosphate glycolaldehydetransferase, EC 2.2.1.1) is a key enzyme of the nonoxidative pentose phosphate pathway (PPP), using glucose to generate ribose-5-phosphate (R5P). R5P is then used in the biosynthesis of nucleic acids and certain coenzymes involved in nucleic acid synthesis.¹⁻³ TK catalyzes cleavage of a C-C bond in ketose (donor substrate) and subsequent transfer of the 2-carbon fragment to aldose (acceptor substrate). This transketolase activity requires thiamine diphosphate (ThDP) and bivalent cations such as Ca²⁺ or Mg²⁺ as cofactors.⁴

Glucose carbons are the main source of de novo nucleic acid synthesis in tumor cells, as revealed by studies on labeled glucose carbons with isotope recovered from tumor cell RNA and DNA.⁵

The pentose cycle for generating RNA ribose consists of an oxidative PPP and a nonoxidative PPP. The latter is regulated by TK. The nonoxidative PPP contributes more than 70% of nucleic acid ribose synthesis in tumor cells.⁵⁻⁸ The essential role of TK in the nonoxidative PPP and the importance of the nonoxidative pentose cycle in nucleic acid synthesis in tumor cells suggest that inhibition of TK activity and consequent inhibition of the intensive glucose using the nonoxidative pathway selectively in tumor cells may provide a novel approach for cancer therapeutics.

Several synthetic compounds and natural products have been reported to inhibit TK enzyme activity in vitro, inhibit cell proliferation, or suppress tumor growth in mice models as a result of reduction of TK activity. *p*-hydroxyphenylpyruvate, a natural cellular metabolite and analog of the donor substrate of TK, was shown to act as a reversible and competitive inhibitor in the in vitro TK activity assays.⁹ Administration of oxythiamine, an analog of the TK cofactor thiamine, resulted in suppression of tumor cell proliferation in several in vitro and in vivo tumor models.¹⁰ Genistein, a plant isoflavonoid with tumor growth inhibition potential, targets glucose carbon incorporation into nucleic acid ribose through the nonoxidative steps of the pentose cycle.¹¹ Fermented wheat germ (trade name: Avemar) had a marked inhibitory effect on metastasis formation in tumor-bearing animals¹² and in clinically advanced colorectal cancers after chemotherapy and surgery.¹³ Stable iso-

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tope studies indicate that Avemar is a powerful inhibitor of de novo nucleic acid synthesis primarily through nonoxidative PPP in pancreatic adenocarcinoma cells.¹⁴ However, these synthetic compounds or natural products are either not highly specific for TK or are weak inhibitors of TK. Here, for the first time, we report identification of 2 novel small-molecule inhibitors through high-throughput screening (HTS) of library compounds using recombinant human transketolase. These compounds specifically inhibit human transketolase activity and suppress proliferation of colon cancer cells, SW620 and LS174T, and the pancreatic adenocarcinoma cell line, MIA PaCa-2.

MATERIALS AND METHODS

Reagents

Ribose-5-phosphate (R5P), thiamine diphosphate (ThDP), $MgCl_2$, NaCl, imidazole, Tris, dithiothreitol (DTT), nicotinamide adenine dinucleotide (NADH), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and glyceraldehyde-3-phosphate (GDH3P) were purchased from Sigma (St. Louis, MO), and xylulose-5-phosphate (X5P) was purchased from the Institute of Organic Chemistry, TU Darmstadt (Darmstadt, Germany). Triosephosphate isomerase (TPI, EC5.3.1.1) and glycerol-3-phosphate dehydrogenase (G3PDH, EC 1.1.1.8), prepared from rabbit muscle, were purchased from Roche Diagnostics (Indianapolis, IN).

TK gene cloning, expression, and protein purification

The human TK cDNA was amplified using primer pairs 5'-gatccatattggagagctaccacaagcctgac-3' and 5'-gactctcagctaggccttggtgatgagccctcac-3' from a human liver QUICK-Clone cDNA library (Clontech, Palo Alto, CA). These primers incorporated *NdeI* and *XhoI* restriction enzyme sites (italicized above) at the 5'- and 3'-ends of the amplified gene accordingly for precise insertion downstream of the T7 promoter in the pET19b expression vector (Novagen, Madison, WI). The sequence of the cloned insert was verified and matched with that of the human TK gene sequence in GenBank (GenBank no. HSU55017). The recombinant pET19b construct containing the TK gene was transformed into *Escherichia coli* Rosetta™ (DE3) pLysS (Novagen). The optimal level of soluble His₆-TK protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C for 4 h. Crude cell lysate was prepared in lysis buffer (20 mM Tris [pH 7.5], 5 mM imidazole, 5 mM β-mercaptoethanol, 500 mM NaCl, and 1% CHAPS) by freezing (dry ice, 10 min) and thawing (37 °C, 5 min) 3 times. Soluble protein fractions were obtained by centrifugation of the cell lysate at 12,000g for 30 min at 4 °C. His₆-TK protein was purified with Ni-NTA resins (Qiagen, Valencia, CA) according to the manufacturer's instructions with elution buffer containing 200 mM imidazole. Imidazole and salt were subsequently removed by dialysis against 0.1 M Tris (pH 7.5) and 10% glycerol overnight at 4 °C. Purified protein was quantified by

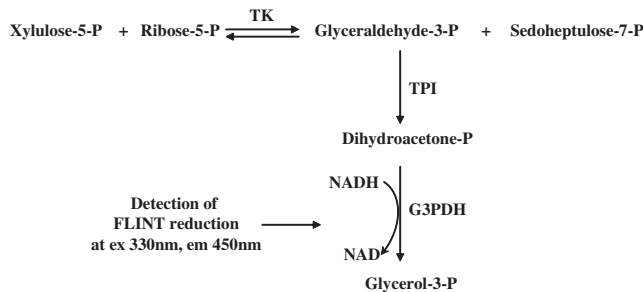


FIG. 1. Diagram showing TK assay reaction. The TK assay involves a primary reaction catalyzed by TK and coupled reactions catalyzed by TPI and G3PDH. TK enzyme activity is measured by FLINT reduction due to concomitant oxidization of NADH with excitation at 330 nm and emission at 450 nm.

the BCA protein assay kit (Pierce Chemicals, Dallas, TX) and visualized on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The purified enzymes were stored at –20 °C in 40% glycerol.

In vitro TK assay

TK activity was measured by a coupled-reaction system based on fluorescent intensity (FLINT) measurement of changes in NADH concentration (Fig. 1).⁴ X5P and R5P were used as substrates and were converted by TK to the products GDH3P and sedoheptulose-7-phosphate (S7P). In the coupled reactions, GDH3P was converted to dihydroacetone phosphate by the enzyme TPI, which in turn was converted to glycerol-3-phosphate (G3P) by the enzyme G3PDH with the concomitant oxidation of NADH. The disappearance of NADH proportional to TK activity was measured by the decrease of FLINT with excitation at 330 nm and emission at 450 nm. Enzyme reactions were conducted on black polypropylene 384-well plates (Greiner) in a volume of 50 μL, and FLINT was recorded as counts per second (CPS) on an Analyst reader (Molecular Devices, Sunnyvale, CA).

Substrate (X5P and R5P) concentration was set at 0.3 mM to be equal to the reported and in-house confirmed K_m value (data not shown) of TK enzyme.¹⁵ Reaction time was set at 2 h for easy and consistent handling of multiple plates with the liquid-handling system (Labcyte, Union City, CA). To optimize assay conditions, concentrations of each of the reaction components were varied individually, and those that gave optimal TK activity were selected. A time course study was then conducted with optimal component concentration to determine reaction kinetics at different concentrations of TK enzyme. The TK enzyme concentration that gave best linear kinetics in a 2-h reaction was selected for the HTS TK assay.

Coupled-reaction assay

Because 2 enzymes, TPI and G3PDH, were included in the coupled-reaction system, a separate coupled-reaction assay was

developed to exclude false positives due to inhibition of the coupled enzymes. In the coupled-reaction assay, the same condition as the regular TK assay was adopted, except that GDH3P was used as substrate and X5P, R5P, or TK enzyme was not included (Fig. 1). Activity of the enzymes in the coupled reaction was measured in a FLINT assay based on reduction of FLINT due to concomitant conversion of NADH to NAD. Assay optimization was focused on selecting an appropriate concentration of the substrate GDH3P that would give a similar kinetics to that of the regular TK assay.

Library compound screening

A total of 64,320 selected samples, including single or pooled compounds, were screened at a final concentration of 5 M. Samples were dissolved in DMSO and dispensed into columns 3 through 22 in 384-well plates at 1.25 L per well. Columns 1 and 2 were used for controls of substrate titration. Columns 23 and 24 were used for controls of reactions with or without TK enzymes (enzyme control [EC] or no enzyme control [NEC]). Substrate solutions containing X5P, R5P, ThDP, MgCl₂, and NADH were dissolved in H₂O to twice the final concentration, and 25 L was dispensed into each well of a 384-well assay plate containing the testing samples, using the Labcyte EDR-384S automatic dispenser (Labcyte, Union City, CA). After brief mixing with a PerkinElmer plate shaker, a FLINT reading was taken using an Analyst reader (prereading). The enzyme solution containing DTT and the enzymes TK, TPI, and G3PDH was prepared in Tris buffer, pH 7.5, in twice the final concentration, and 25 L was dispensed into each well using a Labcyte EDR-384S automatic dispenser. After a 2-h incubation, a FLINT reading (postreading) was taken. Ten 384-well compound plates (3200 samples) were screened per assay.

HTS data analysis

Data analysis was conducted using IDBS XLFit (ID Business Solutions Ltd., Emeryville, CA) or Accelrys Cheminformatics Suite (Accelrys, San Diego, CA). Background fluorescence readings were deducted from the final reading before the calculation of percentage inhibition (%INH). NECs were designated as 100%INH, and ECs were designated as 0%INH. Parameters, including signal over background (S/B), Z',¹⁶ percent signal reduction, and %INH, were calculated according to the following formula:

$$S/B = NEC_{post} / EC_{post}$$

$$Z = 1 - (3 \cdot SD \text{ of } NEC_{post} + 3 \cdot SD \text{ of } EC_{post}) / (NEC_{post} - EC_{post}),$$

$$\% \text{ Signal reduction} = 100 \cdot (NEC_{post} - EC_{post}) / NEC_{post}$$

$$\%INH = 100 \cdot ((S_{post} - EC_{post}) - (S_{pre} - NEC_{pre})) / (NEC_{post} - EC_{post}),$$

where SD is the standard deviation, NEC_{post} is the CPS of NEC from postreading, EC_{post} is the CPS of EC from postreading, S_{post} is the CPS of samples from postreading, S_{pre} is the CPS of samples from prereading, and NEC_{pre} is the CPS of NEC from prereading.

Hit confirmation and IC₅₀ determination

Compounds that displayed more than 50%INH in HTS were selected as primary hits. Inhibition potency of primary hits was confirmed by the concentration response TK assay and the concentration response coupled-reaction assay. Compound serial dilutions were performed in 96-well polypropylene plates (Greiner) using MultiprobeII (Parkard). Diluted compound samples in 96-well plates were then transferred to 384-well assay plates (four 96-well plates to one 384-well plate), 1.25 L each sample, using Biomek FX (Beckman Coulter, Fullerton, CA) or Labcyte EDR-384 multi-autodispenser. Again, columns 1 and 2 were used for controls of substrate titration and columns 23 and 24 for EC and NEC as described above. Identically replicated assay plates containing serially diluted compound samples were tested in both the TK assay and the coupled-reaction assay. The serially diluted compounds were tested at final concentrations ranging from 0.05 to 25 M. A prereading was taken before the reaction started, and a postreading was taken after the reaction was completed. The %INH was calculated by the formula as described in the HTS data analysis section. IC₅₀s were determined using IDBS XLFit. Compounds displaying IC₅₀ less than 5 M in the TK assay and an IC₅₀ ratio of the coupled-reaction IC₅₀/TK assay IC₅₀ > 3 were selected for follow-up studies.

Cell proliferation assay

The colon cancer cell line SW620 was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (Hyclone), and antibiotics (100 U/mL penicillin and 100 g/mL streptomycin). The colon adenocarcinoma cell line LS174T was grown in DMEM supplemented with 2 mM L-glutamine, 10% fetal bovine serum, nonessential amino acids, and antibiotics (100 U/mL penicillin and 100 g/mL streptomycin). The pancreatic adenocarcinoma cell line MIA PaCa-2 was purchased from ATCC (ATCC CRL1420) and grown in DMEM supplemented with 10% fetal bovine serum, 2.5% horse serum (Life Technologies, Inc.), 4 mM L-glutamine, and antibiotics (100 U/mL penicillin and 100 g/mL streptomycin). Cells were cultured at 37 °C under 5% CO₂ and 95% humidity and passed by using 0.25% trypsin no more than 10 times prior to use in the study.

The CyQuant Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR) was used for the cell proliferation assay based on fluorescent staining of cellular nucleic acids. Cells were seeded into 96-well flat-bottom tissue culture plates 1 day before testing to allow attachment to the bottom overnight. For proliferation with SW620 and LS174T cells, 4000 cells per well were seeded, and for MIA PaCa-2, 5000 cells per well were seeded. Compounds were serially diluted 4-fold with culture medium, starting with the highest concentration at 10 M to the lowest at 0.01 M. Each sample was tested in triplicate. For every plate, cells grown in culture medium with 1% DMSO were included as no-compound controls. Cells were incubated with compounds or 1% DMSO in 100 L of

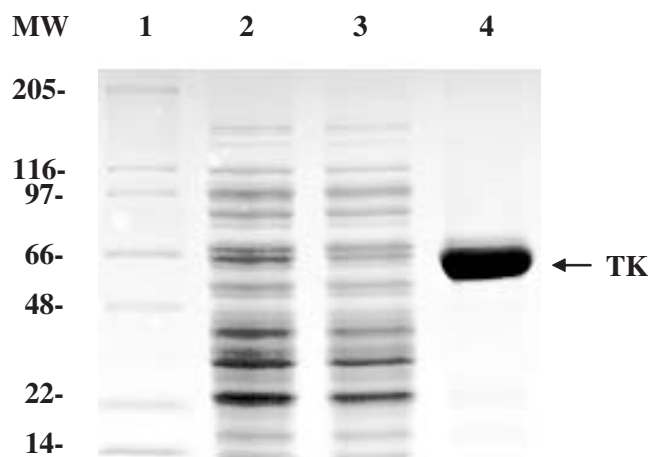


FIG. 2. Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showing purification of recombinant TK. Soluble, insoluble, and Ni-NTA purified fractions of *Escherichia coli* expressing recombinant TK were mixed with 6 \times loading buffer and subjected to SDS-PAGE (10%). Proteins were stained with Coomassie blue following SDS-PAGE. Lane 1: Molecular weight marker; lane 2: soluble fraction of IPTG-induced *E. coli* lysate; lane 3: insoluble fraction of lysate; lane 4: Ni-NTA column purified recombinant TK. Molecular weight in kD is indicated to the left.

culture medium for 4 days (designated as day 4). One plate was collected at the time when compound testing started (designated as day 0) and used as background control. At the end of compound testing, cells from both day 0 and day 4 were lysed by 1 cycle of freeze (-80°C) and thaw (room temperature). CyQuant reagents were added (100 μL /well) following the manufacturer's instructions. Fluorescent intensity was read with excitation at 485 nm and emission at 535 nm by Tecan Ultra (Tecan, USA). GI_{50}s were calculated using GraphPad Prism (GraphPad Software, San Diego, CA).

RESULTS

Expression and purification of TK

Human TK enzyme protein was cloned and expressed in *E. coli* as a fusion protein bearing a His epitope tag.¹⁷ This protein was purified with the Ni-NTA column. One liter of *E. coli* culture produced 5 to 10 mg of purified His₆-TK protein. Purified protein was analyzed by 10% SDS-PAGE and subsequent staining with Coomassie blue. A clear band was observed corresponding to molecular weight consistent with expected TK protein size with His-tag (68.7 kD) from the published cDNA sequence (Fig. 2, lane 4). This protein was used for TK assay development, HTS, and hit confirmation assays.

TK assay development and optimization

TK activity was measured by following disappearance of NADH in the coupled-reaction system. To accurately reflect TK

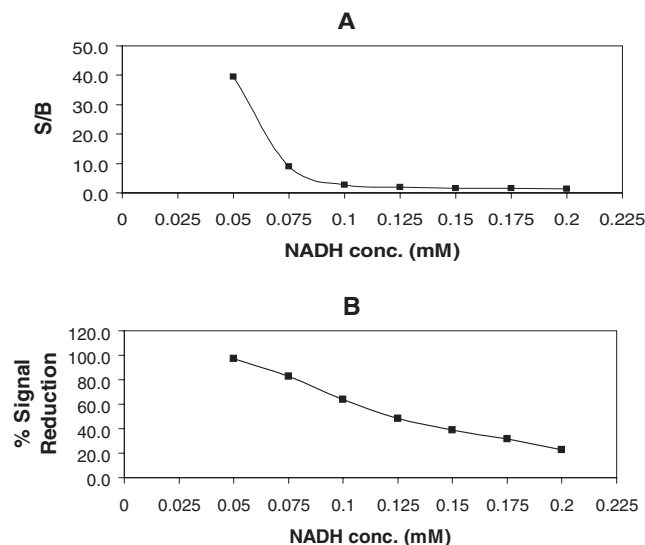


FIG. 3. Graphs showing (A) signal to background (S/B) and (B) percent signal reduction in TK assays with different concentrations of NADH. TK assays were performed with different concentrations of NADH ranging from 0 to 0.2 mM in 0.1 M Tris (pH 7.5), 0.3 mM X5P and R5P, 0.1 mM tThDP, 1.2 mM MgCl_2 , 1 $\mu\text{g}/\text{mL}$ TPI, 3 $\mu\text{g}/\text{mL}$ G3PDH, and 15 nM TK in a 2-h reaction at room temperature. S/B was calculated using the formula described in Materials and Methods. The y-axis represents (A) S/B or (B) percent signal reduction, and the x-axis represents NADH concentrations in mM.

activity, input of NADH must be present within the appropriate concentration range to ensure that the FLINT readout is in the linear response range. An NADH dose-response curve was obtained by determining FLINT measurements at different concentrations of NADH ranging from 0 to 0.5 mM. It was found that the FLINT readout (CPS) remained linear until NADH concentrations exceeded 0.2 mM. Therefore, the input concentration of NADH in the TK assay should be less than 0.2 mM.

To identify the optimal input level of NADH, different concentrations of NADH were tested in the TK assay. The TK assay under different NADH concentrations showed that an S/B of over 5 could only be achieved when NADH concentrations were lower than 0.075 mM (Fig. 3). At this concentration, 82% of NADH was depleted, suggesting that 0.075 mM NADH was also near the lowest concentration possible without complete exhaustion of NADH. Therefore, NADH concentration was set at 0.075 mM for the TK assay.

Optimal concentrations for other reaction components were then determined with 0.075 mM NADH in the TK assay in a 2-h reaction. To ensure that the TK reaction in the coupled-reaction system was the rate-limiting step, the enzymes in the coupled reaction were used at high concentrations. The final conditions used in the assay were 0.3 mM X5P and R5P, 0.075 mM NADH, 1.2 mM MgCl_2 , 0.1 mM ThDP, 2 mM DTT, 1 $\mu\text{g}/\text{mL}$ TPI, and 3 $\mu\text{g}/\text{mL}$ G3PDH in 0.1 M Tris, pH 7.5. A time course study under above optimized conditions was conducted to determine reaction kinetics

Identification of Transketolase Inhibitors

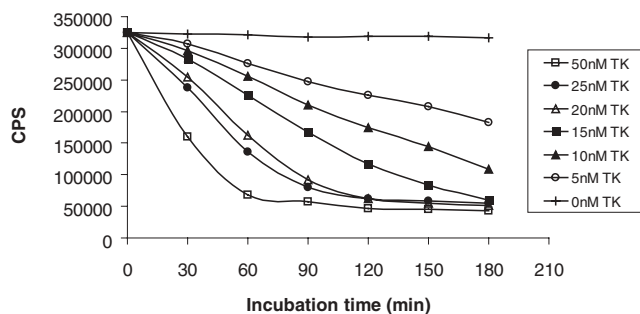


FIG. 4. Time course of TK reaction at optimal conditions at different concentration of TK. TK assays were performed in 0.1 M Tris (pH 7.5), 0.3 mM X5P and R5P, 0.075 mM NADH, 0.1 mM ThDP, 1.2 mM MgCl₂, 1 g/mL TPI, 3 g/mL G3PDH, and different concentrations of TK enzymes ranging from 0 to 50 nM at room temperature. FLINT was measured at 0, 30, 60, 90, 120, 150, and 180 min after the reaction was started. CPS of reactions with different concentrations of TK (y-axis) were plotted against incubation time in min (x-axis).

at different concentrations of the TK enzyme. TK enzyme at a concentration of 15 nM led to a steady linear reduction of signal in a 2-h reaction (Fig. 4). A TK enzyme concentration was thus chosen to be 15 nM for the HTS assay.

Coupled-reaction assay development and optimization

To eliminate false positives that inhibit the coupling enzymes, a separate coupled-reaction assay was developed using GDH3P as a substrate. To define a similar level of NADH conversion, GDH3P was tested at concentrations ranging from 0 to 0.25 mM. Data showed that GDH3P at a concentration of 0.2 mM was able to produce similar levels of NADH conversion, as seen in the TK assay (Fig. 5). However, the conversion of NADH reached a plateau in just 30 min in the separate coupled reaction, and thus a 30-min reaction time for this assay was established to maintain linear kinetics. For the compounds to have the same duration of interaction (2 h) with enzymes in the 2 assays, coupled enzymes were incubated with compounds for 1.5 h before substrate was added to start the 30-min reaction. A prereading was taken before the reaction started, and a postreading was taken after the reaction was completed.

HTS and hit confirmation

A total of 64,320 selected samples were tested at a concentration of 5 μ M. A total of 913 compounds (1.42% of total tested samples) that displayed more than 50%INH were selected as primary hits (Table 1). Selected primary hits were tested for concentration dependence in the 2 assays at final concentrations ranging from 0.05 to 25 μ M. Four independent tests were conducted, and the average of IC₅₀s was obtained. Based on the selection criteria of IC₅₀

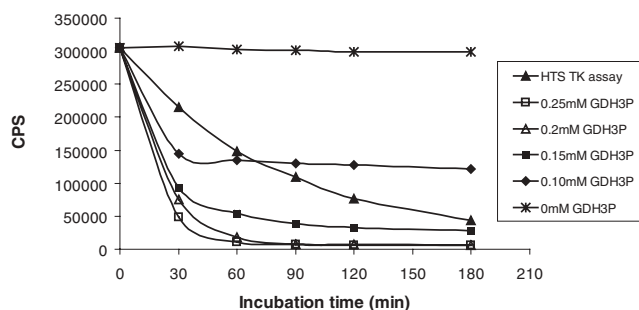


FIG. 5. Time course study of coupled reaction at different concentrations of GDH3P. Coupled reactions were performed with different concentrations of GDH3P in 0.1 M Tris (pH 7.5), 0.1 mM ThDP, 1.2 mM MgCl₂, 0.075 mM NADH, 1 g/mL TPI, and 3 g/mL G3PDH at room temperature. A regular TK assay was performed concurrently as control in a 2-h reaction. FLINT was measured at 0, 30, 60, 90, 120, 150, and 180 min after reaction was started. CPS from reactions with different concentration of GDH3P (y-axis) were plotted against incubation time (x-axis).

< 5 μ M for the TK assay and an IC₅₀ ratio of the coupled reaction/TK assay > 3, a total of 175 compounds (0.27% of total tested samples) were selected as confirmed hits for follow-up studies (Table 1).

Cell proliferation assay

The 175 confirmed hits identified by an in vitro biochemical assay were tested on colon cancer cell lines SW620 and LS174T, as well as the pancreatic carcinoma cell line MIA PaCa-2, for their inhibitory effect on cell proliferation. These 3 cell lines were selected because TK mRNA expression was elevated in SW620 and LS174T and because the TK inhibitor, oxithiamine, inhibited MIA PaCa-2 cell proliferation.^{5,18} Compounds were tested in a concentration response format at concentrations ranging from 0.01 to 10 μ M. Of the compounds, 54 of 175 (0.08% of total tested library compounds) showed a consistent inhibitory effect on cell proliferation in the 3 cancer cell lines (Table 1).

Scaffold selection

Lipinski's "rule of 5,"¹⁹ a widely accepted guideline for compound library design and hit and/or lead selection principles in drug discovery industry, was used for further evaluation of selected hit compounds. Among the 54 confirmed hits demonstrating TK inhibition activity in biochemical assays as well as cell proliferation inhibition assays, 2 compounds were found to have high selectivity for TK (no or low inhibition on other targets; data not shown), novelty of structure, drug-like properties, and acceptable inhibitory activities (Table 2). These 2 compounds showed specific inhibition of TK enzyme activity in biochemical assays and cell proliferation in 3 tested cancer cell lines with IC₅₀ and GI₅₀ in the

Table 1. Statistics of High-Throughput Screening (HTS) and Hit Selection

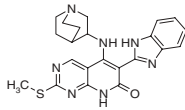
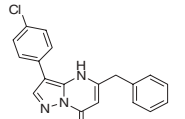
Compounds/Hits	Number	% of Total Tested Samples
Tested compounds	64,320	
Primary hits from HTS (> 50 %INH)	913	1.42
Confirmed hits (TK IC ₅₀ < 5 M, IC ₅₀ ratio CR/TK > 3)	175	0.27
Hits demonstrating cell proliferation inhibition (GI ₅₀ < 10 M)	54	0.08

low M range. These 2 scaffolds were selected for further hit-to-lead studies.

DISCUSSION

It is well established that tumor cells intensively recruit glucose carbons and are exceptionally dependent on glucose metabolism due to elevated energy requirements and intensive de novo nucleic acid, lipid, and amino acid synthesis. Recent studies analyzing glucose metabolic pathways using biological mass spectrometry have shown that several chemicals and natural products inhibit cell proliferation or tumor growth through regulation of glucose intermediate metabolism targeted at the oxidative PPP, the nonoxidative PPP, or both.^{6,11-14,20,21} The tyrosine kinase inhibitor genistein inhibits tumor growth primarily through the regulation of glucose metabolism, specifically targeting the nonoxidative PPP.¹¹ Avemar inhibits metastasis formation also primarily through the non-oxidative PPP, and it decreases activities of glucose-6-phosphate 1-dehydrogenase (G6PDH), a key enzyme in the oxidative PPP, as well as TK.^{12-14,21} Although no chemical constituents in Avemar have been isolated and tested experimentally, it is likely that wheat germ agglutinin plays a significant role in the anticarcinogenic effects. On the other hand, it has been suggested that the oxidative PPP is the potential downstream target pathway of Gleevec (STI571 from Novartis AG) via inhibition of the Bcr-Abl tyrosine kinase. G6PDH and another key enzyme controlling oxidative reactions, hexokinase, are both inhibited by Gleevec treatment.²⁰ Methotrexate (MTX), currently in trials for colorectal carcinoma, inhibits the activity of G6PDH and 6-phosphogluconate dehydrogenases, both involved in oxidative PPP.²² All these data suggest that inhibition of the glucose metabolism pathway may contribute significantly to the effects of cell proliferation and/or tumor growth inhibition and that inhibition of the glucose metabolism pathway may potentially be an effective way to treat cancer, a so-called "cancer starvation" strategy. Because the TK-regulated nonoxidative pathway is much elevated and contributes more than 70% to the ribose synthesis in tumor cells,⁵⁻⁸ this would render tumor cells far more sensitive than normal cells to glucose metabolism inhibition and suggest that inhibition of the TK enzyme activ-

Table 2. Structures, IC₅₀s, GI₅₀s, and Physicochemical Properties of 2 Selected Hit Compounds

Structure	TK assay IC ₅₀ [*] (M)	GI ₅₀ [#] (M)		
		SW620	LS174T	MIA Paca-2
	4.0	0.4	0.7	0.4
ClogP = 3.1, H-bond donor = 3, H-bond acceptor = 8, MW = 433.5				
	3.9	0.4	9.3	10.4
ClogP = 3.9, H-bond donor = 1, H-bond acceptor = 4, MW = 335.8				

* Average of 4 independent tests. # Average of 3 independent tests.

ity and the subsequent block of the intensive nonoxidative PPP may be of particular interest in future cancer therapeutics.

However, previously reported inhibitors either do not exhibit high specificity for TK or are weak inhibitors of the TK enzyme. Identification of novel small-molecule inhibitors with high specificity and potency for TK is needed. In an effort of drug discovery for cancer therapeutics targeting TK, we cloned human TK enzymes and expressed active enzyme in *E. coli*. Using this recombinant TK enzyme, we developed and optimized an assay for compound testing in which TK activity was measured by concomitant disappearance of NADH in a coupled-reaction system. Changes of NADH could be quantified by either spectrophotometric absorbance at 340 nm or FLINT with excitation at 330 nm and emission at 450 nm. The FLINT assay was selected for primary assay as we found that the S/B and the Z' factor in spectrophotometric assays were lower than that in the FLINT assay (1.4 of S/B and 0.6 of the Z' factor in the spectrophotometric assay vs. 5 of S/B and 0.8 of the Z' factor in FLINT), and thus the FLINT assay was considered more sensitive and suitable for HTS.

Because the TK assay involved coupled reactions and additional enzyme activities, hits identified in primary screening may be due to inhibition of the coupled-reaction enzymes and not inhibition of the TK enzyme. Therefore, hits identified from this TK assay were subjected to an independent coupled-reaction assay to eliminate false positives. The separate coupled-reaction assay was developed to mimic the conditions of the TK assay. The reduced time for the NADH conversion in the separate coupled reaction to reach a plateau could be explained by all the substrate (GDH3P) being available at the beginning of the reaction, whereas in the TK assay, GDH3P was gradually generated along the duration of the reaction. To confirm that selected hits were inhibiting TK enzyme other than coupled enzymes, IC₅₀s from TK assay were compared with those from the coupled-reaction assay. An IC₅₀ ratio of the coupled reaction/TK assay > 3 was set as the minimum criterion

for a compound to be considered as having a specific inhibitory effect on TK.

Through an HTS campaign of a selected 64,320 library samples with the FLINT assay, we have identified 54 small molecules that specifically inhibit human transketolase and suppress cancer cell proliferation. Among these 54 hits, 2 novel scaffolds were selected for further studies. Selection of these 2 compounds was based on selectivity, unique structures, and ideal physicochemical properties. Further efforts in medicinal chemistry and lead optimization biology are needed to produce more potent and specific inhibitors for TK. Identification of these interesting scaffolds has set the stage for the development of cancer therapy specifically targeting TK and the nonoxidative pathway of glucose metabolism.

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REFERENCES

- Braus GH: Aromatic amino acid biosynthesis in the yeast *Saccharomyces cerevisiae*: a model system for the regulation of a eukaryotic biosynthetic pathway. *Microbiol Rev* 1991;55:349-370.
- Josephson BL, Fraenkel DG: Transketolase mutants of *Escherichia coli*. *J Bacteriol* 1969;100:1289-1295.
- Wood T: *The Pentose Phosphate Pathway*. Orlando, FL: Academic Press, 1985.
- Kochetov GA: Transketolase from yeast, rat liver, and pig liver. *Methods Enzymol* 1982;90:209-223.
- Boros LG, Puigjaner J, Cascante M, Lee PW-N, Brandes JL, Bassilian S, et al: Oxythiamine and dehydroepiandrosterone inhibit the nonoxidative synthesis of ribose and tumor cell proliferation. *Cancer Res* 1997;57:4242-4248.
- Boros LG, Today JS, Lim S, Bassilian S, Cascante M, Lee PW-N: Transforming growth factor beta2 promotes glucose carbon incorporation into nucleic acid ribose through the nonoxidative pentose cycle in lung epithelial carcinoma cells. *Cancer Res* 2000;60:1183-1185.
- Lee PW-N, Byerley LO, Bassilian S, Ajie HO, Clarck I, Edmond J, Bergner E: Isotopomer study of lipogenesis in human hepatoma cells in culture: contribution of carbon and hydrogen atoms from glucose. *Anal Biochem* 1995;226:100-112.
- Macallan DC, Fullerton CA, Neese RA, Haddock K, Park SS, Hellerstein MK: Measurement of cell proliferation by labeling of DNA with stable isotope-labeled glucose: studies in vitro, in animals, and in humans. *Proc Natl Acad Sci USA* 1998;95:708-713.
- Solovjeva ON, Kochetov, GA: Inhibition of transketolase by *p*-hydroxyphenylpyruvate. *FEBS Lett* 1999;462:246-248.
- Rais B, Comin B, Puigjaner J, Brandes JL, Creppy E, Saboureaux D, et al: Oxythiamine and dehydroepiandrosterone induce a G1 phase cycle arrest in Ehrlich's tumor cells through inhibition of the pentose cycle. *FEBS Lett* 1999;456:113-118.
- Boros LG, Bassilian S, Lim S, Lee PW-N: Genistein inhibits nonoxidative ribose synthesis in MIA pancreatic adenocarcinoma cells: a new mechanism of controlling tumor growth. *Pancreas* 2001;22:1-7.
- Hidvegi M, Raso E, Tomoskozi-Farkas R, Paku S, Lapis K, Szende B: Effect of Avemar and Avemar + vitamin C on tumor growth and metastasis in experimental animals. *Anticancer Res* 1998;18:2353-2358.
- Jakab F, Mayer A, Hoffmann A, Hidvegi M: First clinical data of a natural immunomodulator in colorectal cancer. *Hepatogastroenterolog* 2000;47:393-395.
- Boros LG, Lapid K, Szende B, Tomoskozi-Farkas R, Balogh A, Boren J, et al: Wheat germ extract decreases glucose uptake and RNA ribose formation but increases fatty acid synthesis in MIA pancreatic adenocarcinoma cells. *Pancreas* 2001;23:141-147.
- Schenk G, Duggleby RG, Nixon PF: Properties and functions of the thiamin diphosphate dependent enzyme transketolase. *Int J Biochem Cell Biol* 1998;30:1297-1318.
- Zhang J-H, Chung TDY, Oldenburg KR: A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* 1999;4:67-73.
- Schenk G, Duggleby RG, Nixon PF: Heterologous expression of human transketolase. *Int J Biochem Cell Biol* 1998;30:369-378.
- Fang LJ, Du MX, Sim J, Yin Z: Transketolase inhibitors reduce ribose RNA synthesis through the non-oxidative pathway. In preparation.
- Lipinski CA, Lombardo F, Domiy BW, Feeny PJ: Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev* 2001;46:3-26.
- Boren J, Cascante M, Marin S, Comin-Audoux B, Centelles JJ, Lim S, et al: Gleevec (STI571) influences metabolic enzyme activities and glucose carbon flow toward nucleic acid and fatty acid synthesis in myeloid tumor cells. *J Biochem* 2001;276:37747-37753.
- Comin-Audoux B, Boros LG, Marin S, Boren J, Callol-Massot C, Centelles JJ, et al: Fermented wheat germ extract inhibits glycolysis/pentose cycle enzymes and induces apoptosis through poly(ADP-ribose) polymerase activation in Jurkat T cell leukemia tumor cells. *J Biol Chem* 2002;277:46408-46414.
- Babiak RM, Campello AP, Carnieri EG, Oliveira MB: Methotrexate: pentose cycle and oxidative stress. *Cell Biochem Funct* 1998;16:283-293.

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