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Computational Repositioning and Experimental Validation of Approved Drugs for HIF-Prolyl Hydroxylase Inhibition

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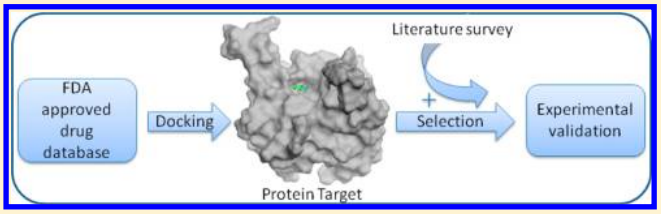
Supporting Information

ABSTRACT: HIF stability and activation are governed by a family of dioxygenases called HIF prolyl-4-hydroxylases (PHDs). It has been identified as a new target to augment the adaptive machinery that governs cytoprotection in disorders associated with ischemia/reperfusion, inflammation, and oxidative stress. In this sense, PHD inhibition has been proposed to mimic, at least in part, the protective effects of exposure to hypoxia. Exploiting drug polypharmacology to identify novel modes of actions for drug repurposing has gained significant attention in the current era of weak drug pipelines. The present work plan aims at giving new purpose to some well-established FDA-approved drugs. Here, we propose that by combining the literature survey, docking, and manual interpretation altogether, we were able to perform virtual screening on FDA-approved drugs to identify potential PHD inhibitors. Upon screening of 1537 marketed drugs, a final set of six hits were selected for experimental testing. All six drugs were divers, and immuno blotting was carried out to evaluate their ability to upregulate HIF in order to validate our hypothesis. Out of the six, three drugs showed significant upregulation of HIF possibly by inhibiting the PHD. It is believed that the appropriate use of the literature survey, docking, manual interpretation, and experimental validation strategy in the drug design process should improve the ability to identify hits and confirm their potential to serve as basis for drug repurposing.



INTRODUCTION

In mammals, one of the most important regulators of oxygen homeostasis is the hypoxia-inducible factor (HIF) complex. HIF coordinates the myriad responses to decreased oxygen tension by promoting compensatory mechanisms acting at the cellular as well as organismal level that include enhancing the oxygen-carrying capacity of blood, decreasing cellular oxygen demand, and increasing glycolysis, to name just a few.

Hypoxia is an effective preconditioning stimulus, and many cellular responses to hypoxia are mediated through a transcription control complex termed HIF.[1] HIF activation results in broad changes of gene expression that can be regarded as adapting the cell, tissue, and organism to conditions of reduced oxygen by initiating multiple survival pathways such as erythropoiesis, angiogenesis, cell survival, neural stem cell growth, inflammation, matrix metabolism, and mitochondrial function.[2−4] Taken together, these observations have suggested that HIF might be involved in mediating the beneficial effects of preconditioning and that pharmacological activation of HIF may be beneficial in diseases associated with oxidative stress, ischemia/reperfusion, and inflammation.[5−10]

HIF stability and activation are governed by a family of dioxygenases called HIF prolyl-4-hydroxylases (PHDs). It has been identified as a new target to augment the adaptive machinery that governs cytoprotection in ischemic condition. Inhibition of PHD activity triggers the cellular homeostatic response to oxygen and glucose deprivation by stabilizing HIF and other proteins.[11] Hence, the development of small molecules that induce HIF-1α activation via inhibition of its degradation pathway has been suggested as a potentially useful therapeutic strategy for the treatment of ischemia/reperfusion injury and inflammatory disorders. In this sense, PHD inhibition has been proposed to mimic, at least in part, the protective effects of exposure to hypoxia.[12]

Although a limited number of inhibitors exist for targeting the PHD, safety is still a serious issue. Only some inhibitors of PHD (FG-4592 and GSK360A) are currently in clinical trial (phase II).[13] Furthermore, there is not even a single FDA-approved PHD inhibitor in the market.

Identifying hit compounds from the FDA drug database has clinical relevance because drug repurposing (also known as drug repositioning, drug reprofiling, therapeutic switching, and drug retasking) has been growing in importance in the past few years as an increasing number of pharmaceutical companies have realized the potential of pre-existing drugs for other medical indications as well (Aspirin and Viagra). Drug repurposing applications are getting more and more attention as it may provide an efficient and effective way to fuel the current drug discovery engines. A major advantage of utilizing approved drugs, given their previously successful clinical trials, is the potential for fast entry into phase II trials for new indications. Therefore, the benefits of increased success rate and decreased costs, resources, and development time make

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drug repurposing an ideal process to kick start productivity in drug development.[13,14]

Keeping in mind the therapeutic significance of this class of inhibitors and our continuing interest in the development of PHD inhibitors, the present study was conceptualized to identify potential PHD inhibitors from the FDA database by virtual screening and validate them experimentally. Hence, the present study aims at giving new purpose to some well-established FDA-approved drugs to treat ischemic disorders by virtue of its PHD inhibitory potentials.

MATERIAL AND METHODS

Receptor X-ray Structure. The 3D coordinates of the X-ray crystallographic structure of human PHD complexed with respective inhibitors or activators (PDB codes: 2HBT, 2G19, 3OUH, 3OUI, and 3OUJ) were selected to generate a receptor grid. Before retrieving ligand from proteins, all structures were prepared by the protein preparation wizard of Schrödinger Suite (Schrödinger LLC., Portland, U.S.A.), and all hetero atoms (except inhibitor and metal ion) were removed from protein files. All water molecules (beyond 3 Å from the inhibitor) and the rest of the chains (except A) were removed from the complex, and the protein was minimized using the OPLS-2005 force field. Further, H atoms were added to the protein to correct ionization and tautomeric states of amino acid residues.[12,15]

Ligand Preparation. All the ligands used were processed in a similar manner. The U.S. Food and Drug Administration-approved drug (hereafter FDA; 1537 molecules) databases were downloaded (http://www.drugbank.ca) and processed to get 3D structures. 3D structures were incorporated into the LigPrep module. The cleaning process was carried out with the following parameters. (1) The force field used was OPLS-2005. (2) All possible ionization states at pH 7.0 ± 2 were generated with an ionizer. (3) The desalt option was activated. (4) Tautomers were generated for all ionization states at pH 7.0 ± 2. (5) Chiralities, when present, were determined from the 3D structure. (6) One low-energy ring conformation per ligand was generated.[16,17]

Structure-Based Virtual Screening. The protein was prepared using protein preparation and refinement tool. For the active site, a grid box centered at the ligand (UN9) was used to accommodate a maximum ligand length of 15 Å. The positional constraint and constraint for Fe^{2+}, Arg383, and H-bond with Tyr329 were applied with grid preparation. Default values were accepted for van der Waals scaling and input partial charges were used. We docked the ligands flexibly, allowing for the flip of the 5- and 6-membered rings, writing out a maximum of one poses per ligand and also enabling the post-docking minimization of the ligands.[12]

The crystal structure of PHD (PDB ID: 2HBT) complexed with an inhibitor, UN9, was taken based on its high resolution (1.60 Å). The inhibitor was sketched and docked by the high-throughput virtual screening (HTVS) mode and extra precision (XP) mode of Glide. The docking conformation was aligned with the original inhibitor conformation to check root mean square deviation (RMSD) in order to confirm the accuracy of docking program.[15]

To identify inhibitors against the above processed PHD, the HTVS mode of Glide was used for the first level screening using the FDA-approved drugs databases. Default parameters were used for docking without any constraints. The top-scored conformations from HTVS docking were subjected to standard precision (SP) docking to rescore the conformations, and finally, all the obtained molecules from SP were subjected to the Glide XP mode. XP docking with receptor flexibility was used for screened out drugs with default settings for some parameters, and no constraints of similarity scoring were applied. XP was used to perform automated docking with full acyclic ligand flexibility, partial cyclic ligand flexibility, and partial protein flexibility in the neighborhood of the protein active site.[18,19]

Cell Culture and Drug Treatment. SH-SY5Y neuroblastoma cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin in a normoxic atmosphere of 5% CO_2 (by vol) in air. Cells were grown up to 70−80% confluency before any experimentation.

Cells in 35 mm dishes were treated with different concentration (200 μM and 600 μM) of drugs for 4 h, and then they were rinsed twice with cold PBS and harvested for Western blot analysis.

Western Blot. Cells were lysed in 100 μL extraction buffer [300 mM sodium chloride, 10 mM Tris (pH 7.9), 1 mM EDTA, 0.1% Nonidet P-40, and 1X protease inhibitor mixture; Roche Applied Science, Basel, Switzerland] for 30 min on ice and centrifuged (3600g at 4 °C for 5 min). The supernatant was used as whole cell extract, and the protein concentration was determined using a protein assay reagent (Bio-Rad). Equal amounts of total cell lysates (50 μg of protein) were resolved on 7.5% SDS-polyacrylamide gels and blotted onto polyvinylidene fluoride (PVDF) membranes. Unspecific binding sites were blocked by incubation in Tris-buffered saline (50 mM Tris/HCl, 150 mM NaCl) containing 0.5% Tween 20 (TBST, pH 7.2) and 5% skimmed milk. Mouse monoclonal anti-HIF-1α (1:500, BD Transduction Labotatories, San Diego, CA) and rabbit anti-β-actin (1:1000, Santa Cruz Biotechnology, Heidelberg, Germany) were served as primary and horseradish peroxidase-conjugated goat antimouse IgG (1:10000, Santa Cruz Biotechnology, Heidelberg, Germany) and goat anti-rabbit IgG (1:10000, Santa Cruz Biotechnology, Heidelberg, Germany) as the secondary antibody. Antibodies were detected with the enhanced chemoluminescence (ECL) kit (GE Healthcare, Munich, Germany). At least six independent Western blots were performed for each experiment and densitometrically adjusted with respect to actin. The densities of desired bands were quantified by the ImageJ software (http://rsb.info.nih.gov/ij/).

Statistical Analysis. The statistical significance between groups was determined on the basis of one-way analysis of variance test followed by Fisher least significant difference Tukey HSD Test (http://vassarstats.net/anova1u.html). Statistical significance was defined at $p \leq 0.05$. All data are expressed as mean ± SEM.

RESULTS AND DISCUSSION

To reduce false predictions, we incorporated multiple PHD−inhibitor complex structures to partially compensate for target flexibility in our computational study. We also filtered the top-ranked hits with literature survey information. The docking-based methods are generally reliable, but combination with the literature survey and manual interpretation altogether may give better results.

Structural Overview of HIF Prolyl-4-hydroxylase. The active site was analyzed by selecting neighbors within 5 Å

around the ligand. The active site contains highly conserved amino acid residues (Arg383, Tyr329, Tyr310, and Tyr303) and Fe^{2+}, essential for binding of ligand to the active site. Arg383 and Tyr329 are responsible for making the salt bridge interaction and H-bonding with the inhibitor, respectively. Tyr310 and Tyr303 are responsible for $\pi-\pi$ stacking and H-bonding with the inhibitor, respectively. The presence of Trp389, Trp258, Met299, and Ilu256 at the opening of the active site renders hydrophobicity.[12]

In Silico Validation of Glide. The reliability of the docking protocol was evaluated by comparing the crystallographic pose of the 2HBT complex structure with the processed model. The crystal structure of PHD (PDB ID: 2HBT) complexed with an inhibitor, UN9, was taken based on its high resolution (1.60 Å). The inhibitor was sketched and docked by the HTVS mode and XP mode of Glide. The docking conformation was aligned with the original inhibitor conformation to check root mean square deviation (RMSD). The docking procedure precisely predicted the experimental result (Figure S1, Supporting Information). This indicates that the Glide docking protocol represents nearly the same binding conformation that is present in native crystal structure.[15]

Selection of Inhibitors. Binding of a ligand to at least one conserved functional residue is likely to interfere with the binding or catalysis of substrate and would result in inhibition of the function of the protein. Out of 1537 molecules from the FDA drug database, 240 hits were initially obtained with the HTVS mode and SP mode. The ligands were allowed to bind with processed PHD in the more precise Glide XP mode, and subsequently, 28 ligands with good binding energy (−24.9 kcal/mol or higher) were selected. These ligands interact with at least one of the experimentally determined conserved functional residues.

The binding modes for 28 compounds identified by Glide XP were ranked according to the information obtained by different scoring constraints. After visual inspection, the most favorable compounds with the best binding modes were selected. On the basis of the knowledge of the existing PHD inhibitors, docking scores, and the active site requirements, we selected 10 compounds out of the 28 highest scoring structures. The six hits with the highest binding affinities were ultimately selected after careful observations, analysis, literature survey, availability, and comparison (Table 1).

Table 1. Docking Score and Other Energy Parameters for Six Selected Drugs

entry name	potential energy	docking score	Glide energy	H-bond
aspartame	14.19	−6.83	−35.54	−1.09
methotrexate	91.20	−6.26	−61.07	−2.43
nalidixic acid	24.38	−6.02	−30.63	0
folic acid	74.34	−5.71	−53.70	−3.35
chloramphenicol	68.44	−5.11	−39.92	−1.54
acetazolamide	29.27	−3.66	−32.91	−1.33

The docking analysis demonstrated that oxygen and nitrogen functionalities in the ligand make a strong ionic interaction with Fe^{2+}. The carboxylic groups interact with Arg383 and Tyr329 through H-bonding. The pentacyclic or hexacyclic rings of inhibitors form a $\pi-\pi$ stacking with benzyl ring of Tyr303 (Figure 1). Further, the presence of a cyclic ring in the ligand assists strong interactions with the hydrophobic opening of the active site and stabilizes it. Also, the combination of these interactions observed in the crystal structure complex (e.g., 2HBT, 2G19, 3OUH, 3OUI, and 3OUJ) strongly supported our docking results.

Structural Analysis of the Inhibition. The docking analysis of six hits indicated that most of the molecules share common intermolecular interactions with the enzyme. All hits (except nalidixic acid) formed a salt bridge and H-bond with the Arg383 and Tyr329 residues, respectively. All six compounds have shown interaction with the Fe^{2+}, and some of them formed $\pi-\pi$ overlaps with Tyr310 (methotrexate, folic acid, aspartame, and nalidixic acid). Hydrophobic interactions were also detected for all hits. Moreover, chloramphenicol has formed H-bonding with Tyr303 also and showed strong interactions (Figure 1).

Effect of Drugs on PHD. To establish the pharmacological characteristics of selected drugs, all six FDA-approved drugs showing a good docking score were considered for in vitro validation by carrying out immuno blotting for HIF-1α upregulation (Table 1). Two concentrations (200 and 600 μM) were used for the study. Out of the six hits, three (folic acid, methotrexate, and chloramphenicol) compounds were able to upregulate HIF-1α significantly, suggesting that they likely inhibited the PHD (2). Moreover, folic acid, methotrexate, and chloramphenicol have good docking scores and desired binding orientation (Figure 1 and Table 1). While other three (acetazolamide, nalidixic acid, and aspartame) upregulated the HIF level in a nonsignificant manner (2). The probable reason may be aspartame gets degraded quickly. Further, acetazolamide had a low docking score, and the binding with PHD was not strong enough to demonstrate significant HIF upregulation. It is well documented that nalidixic acid can act as an iron-chelating agent.[20,21] Many iron-chelating agents have been proven for their potential to upregulate HIF.[22,23] Our computational results showed a good docking score for nalidixic acid and further demonstrated that it interacted with iron (Figure 1). However, Western blot results indicated that nalidixic acid moderately upregulated the HIF level (Figure 2). The possible reason for the observed effect may be that the nalidixic acid neither showed the desired binding orientation nor it did form any salt bridge interaction with Arg383 or H-bond with Tyr329, which is a prerequisite for PHD inhibition (Figure 1 and Table 1).

Although three drugs have not shown significant HIF upregulation, the outcome of the present study is potentially significant. The repurposed molecules are well-established FDA-approved drugs, which can be used directly for their clinical application. Moreover, all the reported drugs have demonstrated their potentials in various disorders associated with oxidative stress, ischemia/reperfusion, and inflammation; the reason for which may be related to their ability to upregulate HIF (Table 2).

These findings indicated that these FDA-approved drugs have some inhibitory potential on PHD and need to be evaluated further in order to confirm their therapeutic potentials. The findings of this study give us some important clues about the probable mechanism of polypharmacological features of these drugs. By combining the in silico reverse identification with experimental approaches, one can predict new targets of any small molecule. Further pharmacological studies including clinical trials are required to explore the full therapeutic potential of these FDA drugs.

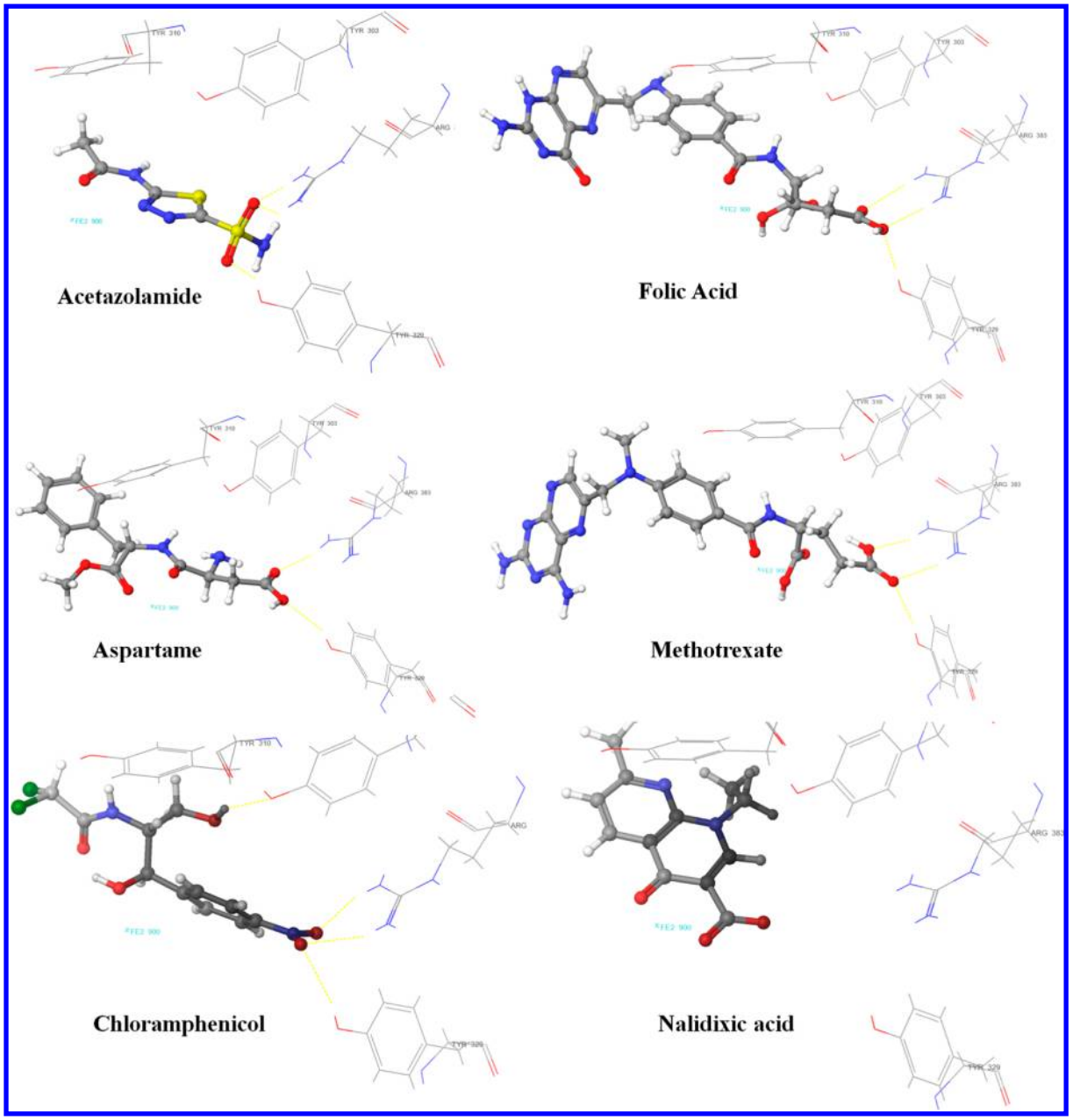

Figure 1. Molecular docking results. Binding orientations of hits in the active site of PHD. All interactive amino acid residues and Fe^{2+} in the active site are shown in normal form, and H-bond interactions are indicated with yellow dotted lines (carbon atoms in gray; oxygen atoms in red; nitrogen atoms in blue; sulfur atoms in yellow).

CONCLUSIONS

FDA-approved drugs are among the most important resources for global drug discovery and development due to the fact that they are abundant in number with unique chemical diversity, and once identified for drug repurposing, they need not go to preclinical testing again.

By combining the literature survey, docking, and manual interpretation altogether, we were able to perform virtual screening on FDA-approved drugs to identify potential PHD inhibitors. The docking analysis demonstrated that all ligands make strong ionic interactions with Fe^{2+}. The carboxylic groups of all ligands (except nalidixic acid) interact with Arg383 and Tyr329 through H-bonding. The active site has a hydrophobic opening, so the presence of cyclic rings in the ligands assist strong interactions and stabilizes them. All six screened out drugs were chemical divers and tested for their PHD inhibitory activity by Immuno blotting in order to validate our computational result. Out of the six, three drugs were shown to significantly upregulate HIF, possibly through PHD inhibition. The result of the present study is potentially significant on the basis of the following considerations. (i) All the hits are well-established FDA-approved drugs, which can be used directly for their clinical application. (ii) The compounds we identified are only the first generation hits from virtual screening against the target site, and the existing library needs checked for their potential.

The knowledge gained from the literature survey and the observations made from structure-based virtual screening, it can be deduced that the combination of molecular docking, manual interpretation, and experimental validation can be more

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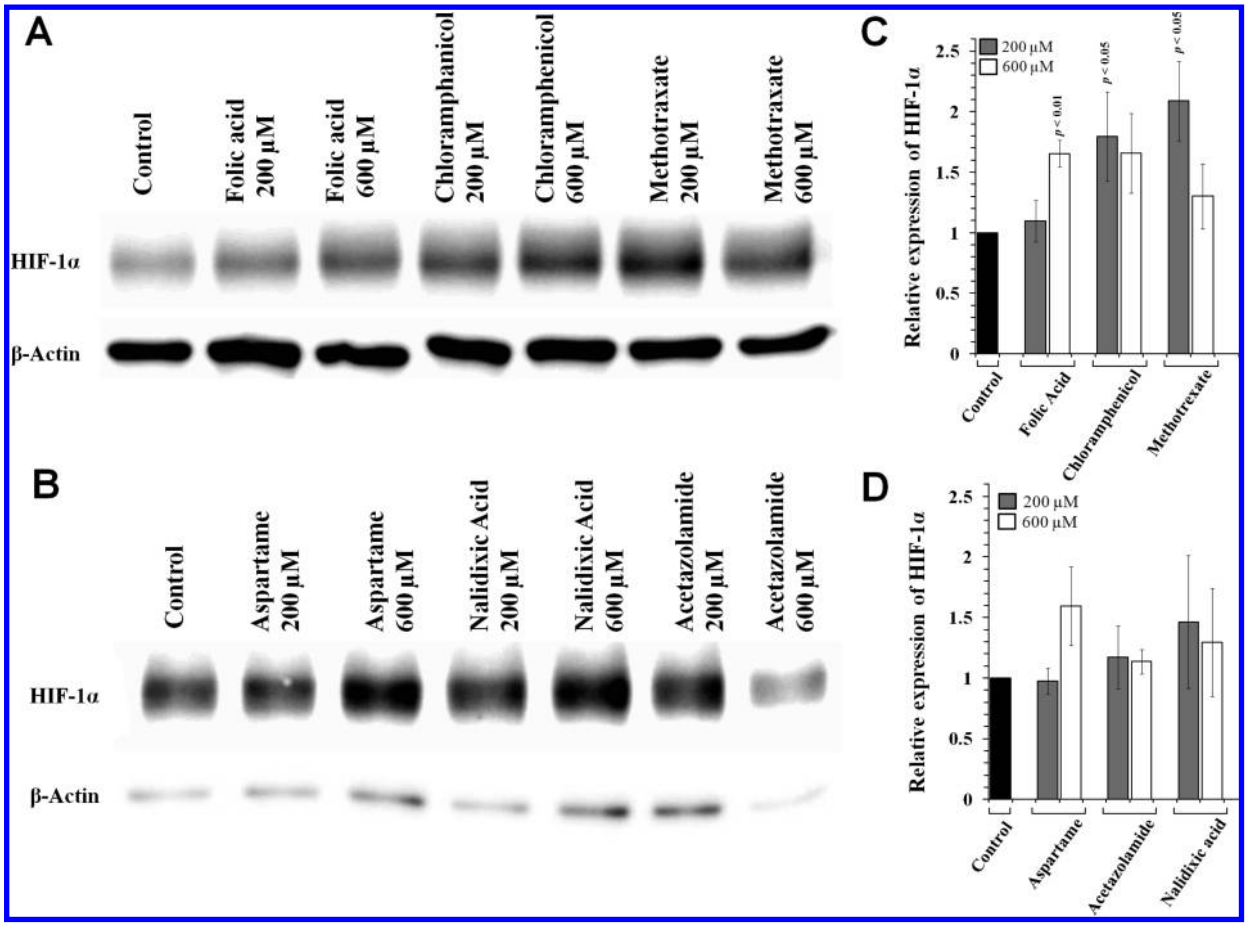

Figure 2. Immuno blotting analysis of HIF-1α expression in the human SH-SY5Y cell line. Cells were treated with a vehicle (C) or a different drug concentration. (A–B) HIF-1α protein levels were analyzed in cell extracts by Western blot analysis, with β-actin serving as loading control. (C–D) Upregulation of HIF-1α was quantified by densitometry. Values were normalized to that of β-actin, and relative expression was compared with the same cell line with different drug concentration vs control. Results shown are representative of six independent experiments, and values are mean ± SEM. Statistical significance was defined at $p \leq 0.05$.

Table 2. Table Listing the Therapeutic Potential of FDA-Approved Drugs against Various Disorders Associated with Oxidative Stress, Ischemia/Reperfusion, and Inflammation

FDA approved drugs	polypharmacological nature of the drug	references
folic acid (vitamin)	prevented neuronal damage in oxygen- and glucose-deprived cells	24, 25
	prevented anxiety-like behavior caused by neonatal hypoxia ischemia	
methotrexate (anticancer)	improves the physical capacity of patients with ischemic heart failure	26–28
	beneficial for treating rheumatoid arthritis and multiple sclerosis	
chloramphenicol (antimicrobial)	cardioprotective against myocardial ischemia/reperfusion injury	29–31
aspartame (artificial sweetener)	angiogenic agent at low doses and induces VEGF	32, 33
	sickled cells reduction in homozygous blood	
acetazolamide (carbonic anhydrase inhibitor)	effective for acute mountain sickness	34, 35
	upregulates HIF by increasing acidosis	
nalidixic acid (antimicrobial)	iron-chelating agent	20, 21

efficient than the traditional approaches alone for discovering polypharmacological features of FDA-approved drugs that may have a great therapeutic impact.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1: Docking protocol (XP and HTVS) validation for PHD by redocking the active compound. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. M.K.T. and R.G.K. conceived and designed the experiments. M.K.T. performed the experiments. M.K.T. and R.G.K. analyzed the data and wrote the paper.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

hypoxia-inducible factor, HIF; HIF prolyl-4-hydroxylases, PHDs; food and drug administration, FDA; root mean square deviation, RMSD; high-throughput virtual screening, HTVS; standard precision, SP; standard precision, SP; Dulbecco's Modified Eagle's Medium, DMEM; polyvinylidene fluoride, PVDF; enhanced chemoluminescence, ECL

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