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Revisiting the role of dihydroorotate dehydrogenase as a therapeutic target for cancer

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ABSTRACT

Identified as a hallmark of cancer, metabolic reprogramming allows cancer cells to rapidly proliferate, resist chemotherapies, invade, metastasize, and survive a nutrient-deprived microenvironment. Rapidly growing cells depend on sufficient concentrations of nucleotides to sustain proliferation. One enzyme essential for the *de novo* biosynthesis of pyrimidine-based nucleotides is dihydroorotate dehydrogenase (DHODH), a known therapeutic target for multiple diseases. Brequinar, leflunomide, and teriflunomide, all of which are potent DHODH inhibitors, have been clinically evaluated but failed to receive FDA approval for the treatment of cancer. Inhibition of DHODH depletes intracellular pyrimidine nucleotide pools and results in cell cycle arrest in S-phase, sensitization to current chemotherapies, and differentiation in neural crest cells and acute myeloid leukemia (AML). Furthermore, DHODH is a synthetic lethal susceptibility in several oncogenic backgrounds. Therefore, DHODH-targeted therapy has potential value as part of a combination therapy for the treatment of cancer. In this review, we focus on the *de novo* pyrimidine biosynthesis pathway as a target for cancer therapy, and in particular, DHODH. In the first part, we provide a comprehensive overview of this pathway and its regulation in cancer. We further describe the relevance of DHODH as a target for cancer therapy using bioinformatic analyses. We then explore the preclinical and clinical results of pharmacological strategies to target the *de novo* pyrimidine biosynthesis pathway, with an emphasis on DHODH. Finally, we discuss potential strategies to harness DHODH as a target for the treatment of cancer.

Contents

Abbreviations: 5-FU, 5-fluorouracil; AML, acute myeloid leukemia; CAD, carbamoyl phosphate synthetase, aspartate carbamoyltransferase, and dihydroorotase; CCLE, Cancer Cell Line Encyclopedia; CNT, concentrative nucleoside transporter; COAD, colon adenocarcinoma; CRMP, collapsing response mediator protein; DHODH, dihydroorotate dehydrogenase; DPM, dipyridamole; ENT, equilibrative nucleoside transporter; ETC, electron transport chain; FMN, flavin mononucleotide; LGG, low-grade glioma; LIHC, liver hepatocellular carcinoma; MAPK, mitogen-activated protein kinase; mTORC1, mammalian target of rapamycin complex 1; OMP, orotidine 5′-monophosphate; PAAD, pancreatic adenocarcinoma; PALA, N-(phosphonacetyl)-L-aspartate; POLD2, DNA polymerase delta 2; PPAN, peter pan homolog; PTEN, phosphatase and tensin homolog; READ, rectum adenocarcinoma; ROS, reactive oxygen species; RRP9, ribosomal subunit RNA processing 9; SLC35, solute carrier 35 family; STAD, stomach adenocarcinoma; TCGA, The Cancer Genome Atlas; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TUFM, Tu translation elongation factor, mitochondrial; UMP, uridine 5′-monophosphate.

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1. Introduction

Cancer is a disease of dysregulated cell growth and metabolism-related enzymes are important targets for drug development (Housman et al., 2014). Targeting enzymes that potentiate a cancer cell's metabolic dependencies presents one of the most successful approaches to halt cell growth. Antimetabolite drugs (*e.g.* 5-fluorouracil, gemcitabine, and methotrexate), which target metabolic liabilities, make up a significant portion of FDA-approved cancer therapies (Kaye, 1998). At least 14 purine- and pyrimidine-based antimetabolites have been FDA-approved for cancer chemotherapy (Parker, 2009). DHODH is a druggable enzyme that plays a vital role in the metabolism of cancer cells. DHODH catalyzes the oxidation of dihydroorotate to orotate, which is essential for the production of uridine monophosphate (UMP) (Munier, Vidalain, Tangy, & Janin, 2013; Reis, Calil, Feliciano, Pinheiro, & Nonato, 2017; Vyas & Ghate, 2011). Inhibition of DHODH induces pyrimidine depletion, thereby starving the cell of the essential nucleotides required to progress through S-phase (Koundinya et al., 2018; Ladds et al., 2018; Mohamad Fairus, Choudhary, Hosahalli, Kavitha, & Shatrah, 2017). Extensive efforts have been made to develop inhibitors of DHODH for cancer therapy, however none to date have gained FDA approval.

In this review, we present compelling evidence in support of DHODH as an essential enzyme for the survival of cancer cells. DHODH, and its relationship to *de novo* pyrimidine metabolism, will be discussed along with factors that influence its regulation and expression. We will show evidence of DHODH's potential clinical relevance and co-expression network using data from The Cancer Genome Atlas (TCGA) and additional published datasets in the context of glioma. Additionally, results using previous DHODH inhibitors in cancer clinical trials and potential improvements for DHODH-targeted therapy will be discussed.

2. The *de novo* pyrimidine biosynthesis pathway and DHODH

DHODH is a vital enzyme in the *de novo* pyrimidine biosynthesis pathway. Through this pathway, cancer cells generate the required substrates for continual DNA replication and protein synthesis without the limitations of nucleotide salvage pathways (discussed below). Pharmacological inhibition of this pathway provides a selective approach to targeting cells undergoing rapid growth. When cells are not preparing for growth, nucleotide demand is primarily maintained *via* nucleotide salvage pathways (Evans & Guy, 2004; Fairbanks, Bofill, Ruckemann, & Simmonds, 1995). Alternatively, cells preparing for proliferation depend on *de novo* nucleotide biosynthesis to fuel nucleotide demands. As a result, enzymes that are a part of the *de novo* nucleotide biosynthesis pathways are frequently overexpressed in cancer to sustain growth and are attractive targets to suppress cancer cell proliferation (Weber, 2001).

In the *de novo* pyrimidine biosynthetic pathway, DHODH catalyzes a committed step and thus presents as a desirable target for halting pathway flux. Overall, the *de novo* pyrimidine pathway generates UMP from glutamine (Fig. 1). Flux through this pathway begins with a large enzymatic complex that catalyzes the first three steps. This complex, known as CAD (an acronym for its domains), is made up of carbamoyl phosphate synthetase, aspartate carbamoyltransferase, and dihydroorotase. The carbamoyl phosphate synthetase domain catalyzes the first reaction and generates carbamoyl phosphate from bicarbonate, ATP, and glutamine or ammonia (Fig. 1, step 1) (Evans & Guy, 2004). The second step is catalyzed by the aspartate carbamoyltransferase domain, converting carbamoyl phosphate into carbamoyl aspartate (Fig. 1, step 2) (Evans & Guy, 2004). The dihydroorotase domain hydrolyzes carbamoyl aspartate into dihydroorotate and generates the substrate for DHODH (Fig. 1, step 3). DHODH oxidizes dihydroorotate into orotate (Fig. 1, step 4) and is the only pathway enzyme located in the mitochondria. The final two steps in the pathway are catalyzed by another large enzyme complex known as uridine monophosphate synthetase. This enzyme is comprised of two domains: orotate phosphoribosyltransferase and orotidine 5′-monophosphate decarboxylase (OMP decarboxylase). The orotate phosphoribosyltransferase domain catalyzes the transfer of a phosphoribosyl group to orotate (Fig. 1, step 5). A final decarboxylation by the OMP decarboxylase domain generates the uridine monophosphate nucleotide (Fig. 1, step 6). Two enzymes, carbamoyl phosphate synthetase and DHODH that catalyze committed steps, primarily control flux through this pathway (Baumgartner et al., 2006; Evans & Guy, 2004; Lane & Fan, 2015). Inhibition of either enzyme halts flux through the *de novo* pyrimidine pathway, but carbamoyl phosphate synthetase is not expressed in all cancer cells. In fact, carbamoyl phosphate synthetase has been observed to have low expression or is completely downregulated in most liver carcinomas (Liu, Dong, Robertson, & Liu, 2011; Siddiqui, Saboorian, Gokaslan, & Ashfaq, 2002). Therefore, inhibition of DHODH's catalytic activity presents as a more pharmacologically relevant approach to treat cancer (Reis et al., 2017).

DHODH catalyzes two redox reactions: the oxidation of dihydroorotate to orotate and subsequent flavin mononucleotide (FMN) regeneration (Fig. 2). The oxidation of dihydroorotate is carried out *via* a stepwise mechanism with highly conserved residues. This first enzymatic reaction follows a deprotonation of dihydroorotate at the C5 position and a subsequent hydride transfer (from dihydororotate's C6 position) to the FMN cofactor (Fig. 2) (Reis et al., 2017). The catalytic base for the initial C5 deprotonation is likely S215 (Fig. 3), which has been reported as the catalytic base for DHODH in other organisms (Bjornberg, Gruner, Roepstorff, & Jensen, 1999; Liu, Neidhardt, Grossman, Ocain, & Clardy, 2000; Reis et al., 2017). Adjacent residues T218 and F149 are highly conserved as well and may increase the basicity of S215 (Fig. 3C) (Liu et al., 2000). However, their precise roles have not been determined. Additional non-catalytic conserved residues contributing to dihydroorotate oxidation are N212, S214, P216, L221, R222, and Q225. These residues are located on a loop region that may be responsible for substrate/product exchange (Liu et al., 2000). The second redox reaction, resulting in regeneration of FMN, requires ubiquinone from the mitochondrial electron transport chain (ETC) (Fig. 2) (Reis et al., 2017). FMN is thought to bind with DHODH's G119 and V282, however the catalytic mechanism of FMN regeneration from ubiquinone is not well understood (Liu et al., 2000). $FMNH_2$ is perceived to undergo multiple single-electron transfers but the exact residues that facilitate this oxidation are not known (Liu et al., 2000; Palfey, Bjornberg, & Jensen, 2001; Reis et al., 2017). Nonetheless, FMN regeneration is necessary for continued DHODH catalysis. Two of the most well-known DHODH inhibitors, leflunomide and brequinar (discussed below), are proposed to act as competitive inhibitors of ubiquinone (Liu et al., 2000). The necessity to regenerate FMN *via* ubiquinone

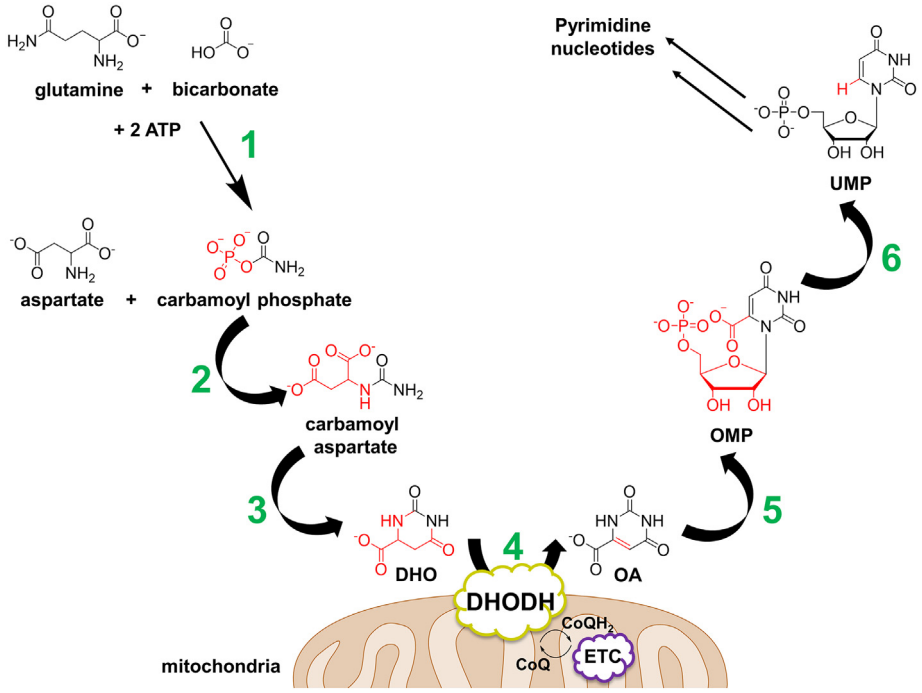


Fig. 1. *De novo* pyrimidine biosynthesis. Numbered steps represent the following enzymes and their catalyzed transformations: (1) Carbamoyl phosphate synthetase catalyzes the conversion of bicarbonate, ATP, and glutamine into carbamoyl phosphate; (2) aspartate carbamoyltransferase catalyzes the conversion of carbamoyl phosphate to carbamoyl aspartate; (3) dihydroorotase catalyzes the conversion of carbamoyl aspartate to dihydroorotate (DHO); (4) dihydroorotate dehydrogenase catalyzes the conversion of dihydroorotate to orotate (OA); (5) orotate phosphoribosyl transferase catalyzes the conversion of orotate to OMP; (6) OMP decarboxylase catalyzes the conversion of OMP to UMP.

may correspond to DHODH localization within the inner mitochondrial membrane. This localization increases exposure to ubiquinone from the mitochondrial ETC.

As a functional ETC is required for DHODH catalysis (Loffler, Jockel, Schuster, & Becker, 1997; Rawls, Knecht, Diekert, Lill, & Loffler, 2000; Zameitat, Freymark, Dietz, Loffler, & Bolker, 2007), DHODH depends on the mitochondrial ETC to generate adequate concentrations of ubiquinone (Fig. 2). Cells lacking a fully functional mitochondrial ETC have an impaired ability to produce UMP (King & Attardi, 1989; Lane & Fan, 2015; Morais, Desjardins, Turmel, & Zinkewich-Peotti, 1988). Without a functional mitochondrial ETC, cells must be supplemented with pyruvate and uridine to support proliferation (King & Attardi, 1989). This phenomenon is presumed to occur due to limited ubiquinone generation for DHODH catalysis. A cell line containing a dysfunctional mitochondrial ETC has been reported to be transformed with alternative ubiquinone oxidative enzymes. Once transformed, the cells were able to sustain growth in uridine- and pyruvate-free media (Perales-Clemente et al., 2008). This result suggests that cells containing a dysfunctional ETC may be indirectly inhibiting DHODH and depleting intracellular pyrimidine nucleotides necessary for growth. Previously, decreased DHODH activity has been observed with small molecule inhibitors of the ETC targeting cytochrome c oxidase (Beuneu et al.,

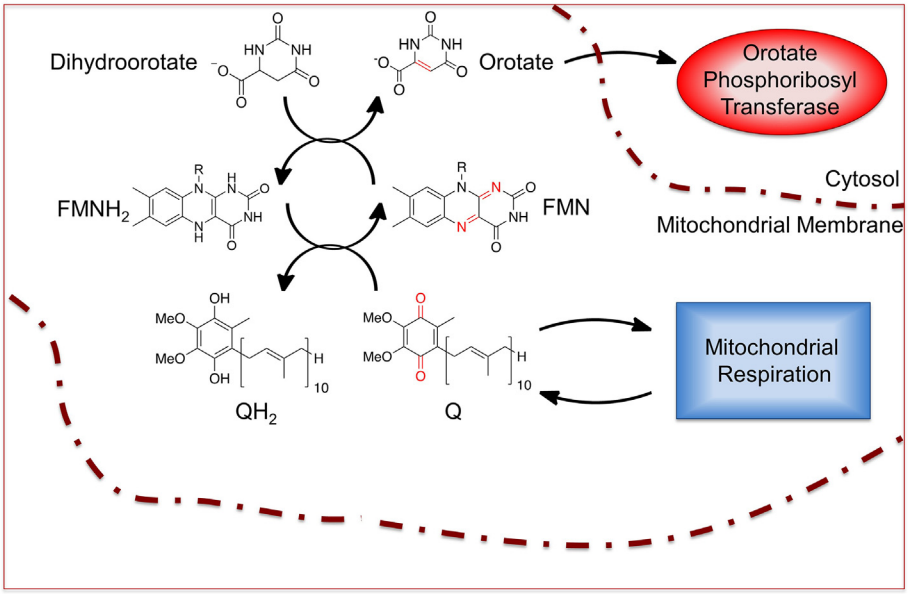


Fig. 2. DHODH couples mitochondrial respiration with *de novo* pyrimidine biosynthesis. FMN (flavin mononucleotide, oxidized) $FMNH_2$ (flavin mononucleotide, reduced), Q (ubiquinone, oxidized), QH_2 (ubiquinone, reduced).

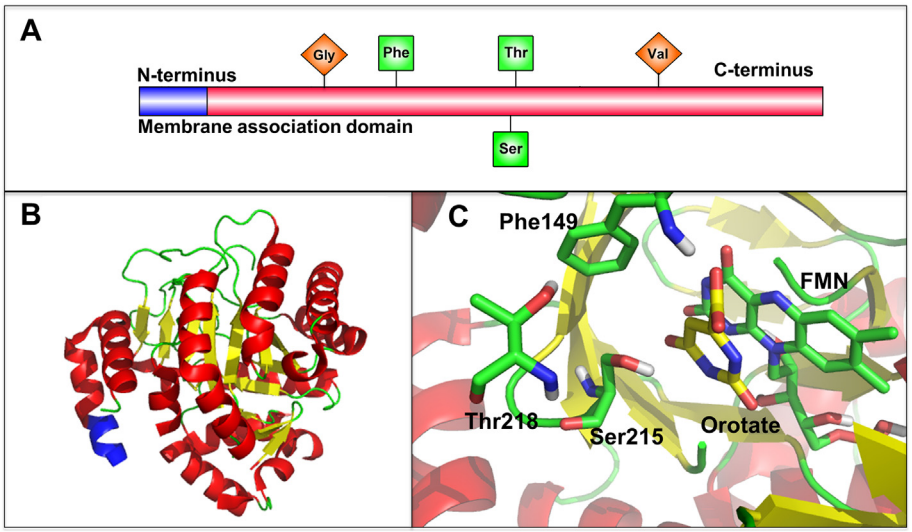


Fig. 3. Structural features of DHODH. A) Overall 2-D structure of DHODH protein with N and C termini highlighted. Highlighted residues play important roles in the active site. Orange diamonds indicate residues that bind FMN, green squares highlight catalytic residues. B) Crystal structure of DHODH (PDB ID: 1D3G). The small N-terminal domain is highlighted in blue. The larger C-terminal domain is color coded by α-helices (red), β-barrels (yellow), and loop regions (green). C) Key residues within the catalytic site and their orientation towards the product orotate (yellow) are highlighted.

2000). Additionally, inhibition of either DHODH or mitochondrial respiration complexes have resulted in similar cellular responses. For example, inhibition of either target induced p53 up-regulation (Khutornenko et al., 2010; Ladds et al., 2018). Collectively, these studies highlight that DHODH catalysis is dependent on a functional ETC or the presence of ubiquinone. This relationship has generated interest in the possible role DHODH may play in reactive oxygen species (ROS) homeostasis.

The connection between DHODH and ROS in cancer is not well understood. Mitochondrial ROS are known to influence cellular proliferation and DHODH-catalyzed oxidation may affect mitochondrial ROS (Diebold & Chandel, 2016; Idelchik, Begley, Begley, & Melendez, 2017). A previous study showed that isolated mitochondria were capable of generating ROS through DHODH and that radical production was diminished by DHODH inhibitors (Hey-Mogensen, Goncalves, Orr, & Brand, 2014). This result implies that DHODH catalysis contributes to elevated ROS levels. However, knockdown of DHODH has been shown to increase the production of ROS and decrease the mitochondrial membrane potential (Fang et al., 2013). Furthermore, DHODH inhibitors were found to decrease the mitochondrial membrane potential (Koundinya et al., 2018). These conflicting results complicate the understanding of DHODH's role in ROS generation. It is possible that inhibitors of DHODH alter redox homeostasis in a context-dependent manner. It was found that cell lines most sensitive to DHODH inhibition consistently generated the lowest amount of ROS (Mohamad Fairus et al., 2017). This data implies that an antioxidant in combination with a DHODH inhibitor may be synergistic or at least additive. However, a DHODH inhibitor, teriflunomide, did not abrogate cell growth when the antioxidant pyrrolidine dithiocarbamate was co-administered (Hail Jr., Chen, Kepa, & Bushman, 2012). These studies highlight a correlation between ROS and DHODH catalysis, but the functional consequences appear to be context-dependent.

3. Regulation of DHODH activity in cancer

Regulation of DHODH activity occurs primarily through activation of *de novo* pyrimidine biosynthesis *via* the CAD complex. When cells are not preparing for growth, flux through the *de novo* pyrimidine pathway is slow and functions to generate RNA nucleotides primarily for protein synthesis (Coleman, Suttle, & Stark, 1977; Evans & Guy, 2004; Jones, 1980). In this state, flux is controlled through product feedback inhibition as high concentrations of uridine inhibit CAD. However, when cells prepare to divide, phosphorylation of CAD alters its affinity for uridine to overcome feedback inhibition (Carrey, Campbell, & Hardie, 1985; Sahay, Guy, Liu, & Evans, 1998). Selective phosphorylation of CAD at specific residues regulates flux through the *de novo* pyrimidine biosynthesis pathway (Huang & Graves, 2003). Flux through the pathway is increased when T456 of CAD is phosphorylated by mitogen-activated protein kinase (MAPK) or mechanistic target of rapamycin 1 complex (mTORC1) *via* S6 kinase (Ben-Sahra, Howell, Asara, & Manning, 2013; Robitaille et al., 2013; Sigoillot, Berkowski, Sigoillot, Kotsis, & Guy, 2003). After sufficient concentrations of nucleotides are reached, protein kinase A phosphorylates S1406 of CAD to down-regulate nucleotide biosynthesis (Kotsis et al., 2007). Overexpression of enzymes controlling CAD phosphorylation, such as MAPK or mTORC1, leads to increased flux through the pathway in cancer. For example, the breast cancer cell line MCF7 overexpressing MAPK kinase causes a 4-fold increase in the rate of *de novo* pyrimidine biosynthesis (Sigoillot, Sigoillot, & Guy, 2004). Modulation of flux through the pathway by mTORC1 has not been confirmed experimentally. In addition to phosphorylation, CAD localization also affects the rate of flux through the *de novo* pyrimidine pathway. Hindrance of CAD nuclear import has been found to decrease the rate of pyrimidine synthesis by 21% and decrease nucleotide concentrations by nearly 60% (Sigoillot et al., 2005). However, it is unclear how and why CAD localization affects *de novo* pyrimidine biosynthesis. Nonetheless, CAD's phosphorylation and localization play a significant role in regulation of DHODH activity.

Beyond phosphorylation, cancer cells may alter pyrimidine biosynthesis through the activation of the proto-oncogenic transcription factor MYC. MYC is a master regulator of many different pathways and has significant influence on the expression of nucleotide metabolism genes. Previous studies have shown that overexpression of MYC significantly increased expression of nucleotide metabolism enzymes, including DHODH, which was validated as a direct MYC target gene (Liu et al., 2008). Additionally, shRNA knockdown of *MYC* decreased the expression of nucleotide metabolism genes and lowered the intracellular concentrations of nucleotides (Mannava et al., 2008). These results demonstrate MYC's control over DHODH expression. Surprisingly, inhibition of DHODH by brequinar or teriflunomide down-regulated MYC expression (Dorasamy, Choudhary, Nellore, Subramanya, & Wong, 2017). However, this may be context-dependent as leflunomide (the pro-drug of teriflunomide) demonstrates no effect on MYC expression, possibly due to insufficient conversion of leflunomide to teriflunomide

(O'Donnell et al., 2012), but this was not confirmed experimentally. Nonetheless, while these conflicting results complicate the understanding of MYC and DHODH's relationship, nucleotide biosynthesis is among the many pathways MYC activation influences to facilitate cellular proliferation. This is a unique relationship as other transcription factors, such as those in the E2F family, do not appear to increase expression of DHODH (Bester et al., 2011). It is possible that MYC and DHODH expression are linked through glutamine metabolism because MYC increases glutamine flux, which is the first substrate in *de novo* pyrimidine biosynthesis (Hsieh, Walton, Altman, Stine, & Dang, 2015).

The relationship between glutamine metabolism and *de novo* pyrimidine biosynthesis is becoming better understood. Low glutamine concentrations have been demonstrated to hinder S-phase progression, presumably by limiting the precursor metabolite for nucleotide production (DeBerardinis & Cheng, 2010; Gaglio, Soldati, Vanoni, Alberghina, & Chiaradonna, 2009). This is in agreement with the recent finding that DHODH inhibitors induce a decrease in the steady-state level of glutamine (Koundinya et al., 2018), and DHODH inhibition is known to cause S-phase arrest. High glutamine concentrations may increase the rate of *de novo* nucleotide synthesis, however, it does not appear that cancer cells increase glutamine uptake to solely fuel *de novo* pathways (DeBerardinis & Cheng, 2010; Wise & Thompson, 2010). A significant disparity exists between the rates of glutamine uptake and nucleotide biosynthesis, suggesting that glutamine is utilized by far more pathways than nucleotide biosynthesis alone (DeBerardinis & Cheng, 2010). Nevertheless, glutamine flux may be an indicator of cell sensitivity to DHODH inhibition. Interestingly, cells with a mutant phosphatase and tensin homolog (PTEN) tumor suppressor were observed to possess increased glutamine metabolism and were sensitive to DHODH inhibition (Mathur et al., 2017). While more studies are needed, these results suggest that DHODH inhibitors may be useful in cells with mutant PTEN and possibly increased glutamine metabolism.

4. DHODH and cell differentiation

Beyond directly halting cell growth, DHODH has been implicated as a target to induce cellular differentiation. Previously, DHODH inhibition was shown to induce cell differentiation in neural crest (White et al., 2011) and leukemic cell lines (Sykes et al., 2016). In leukemic cells, DHODH inhibitors, such as brequinar, were observed to decrease the number of self-renewing cells *in vivo* (Sykes et al., 2016). Recently, a novel class of DHODH inhibitors were found to induce myeloid differentiation in AML cell lines, which led to cytotoxicity of the differentiated cell population (Sainas et al., 2018). Though further studies are needed to fully understand how DHODH inhibitors induce this effect, these findings have significantly increased the interest in DHODH-targeted therapy for cancer. In fact, two newly patented DHODH inhibitors are in clinical trials for the treatment of AML (Table 1); one from Aslan Pharmaceuticals (ASLAN003, Phase II, NCT03451084) and the other from Bayer (BAY2402234, Phase I) (Gradl et al., 2018).

While the mechanism of DHODH inhibition-induced differentiation is not fully understood, this phenomenon may be caused by pyrimidine depletion. Pyrazofurin, an inhibitor of OMP decarboxylase, was able to induce differentiation and suggests pyrimidine depletion as a potential mechanism (Sykes et al., 2016). Additionally, differentiation caused by DHODH inhibition was rescued in the presence of uridine (Sainas et al., 2018). A possible link between pyrimidine depletion and cellular differentiation may be transcriptional elongation. The PHD-finger Phf5a is known to modulate transcriptional elongation of genes involving cell differentiation (Strikoudis et al., 2016). Pyrimidine depletion has been observed to inhibit transcriptional elongation of tumorigenic genes (Tan et al., 2016), and in fact, direct inhibition of DHODH produced a similar effect in melanoma cells (White et al., 2011). However, the connection between DHODH and cellular differentiation is still not well understood. Despite this, considerable interest exists for DHODH-targeted therapy as a potential option to induce cellular differentiation.

5. Relevance of DHODH in cancer

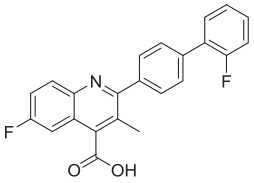
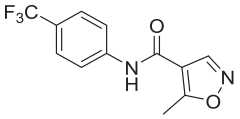
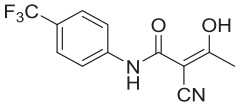
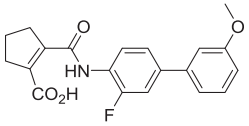
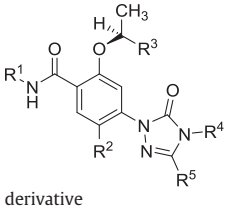
DHODH's relevance in cancer was recognized nearly six decades ago when Smith et al. noted elevated DHODH activity in leukemic cells (Smith, Baker, & Sullivan, 1960; Smith Jr. & Baker, 1959). Following that observation, several investigations focused on DHODH that culminated in the discovery and clinical evaluation of brequinar, a potent human DHODH inhibitor (Dexter et al., 1985; Schwartsmann et al., 1988). However, brequinar failed to produce objective responses in clinical trials (discussed in further detail below), leading to questions about the viability of DHODH inhibition as a therapeutic approach. Recent reports have revisited the link between DHODH inhibition and antiproliferative effects on cells. However, no studies have thoroughly evaluated the clinical relevance of DHODH in cancer. To address this gap in understanding, we analyzed data from several different *in vitro* and *in vivo* profiling projects to provide an unbiased summary of the potential significance of DHODH in cancer.

Cell growth is suppressed when clinically relevant anticancer targets are knocked down. The Achilles Project (Aguirre et al., 2016; Cowley et al., 2014; Tsherniak et al., 2017) evaluates large-scale cellular responses in the presence of shRNA across a catalogue of cancer cell lines and has been utilized to gauge the importance of DHODH in cancer cells. Changes in cell viability were averaged for each cell line and then ranked by essentiality; a negative shRNA score indicates that a cell line responded poorly (*i.e.*, showed growth inhibition) to treatment with shRNA. Achilles' project results (Fig. 4A) depict a resounding negative response of cancer cell lines to shRNA knockdown of *DHODH*. The results suggest that DHODH inhibition correlates with decreased cell growth in most cancer cell lines. Cell lines that were most sensitive to *DHODH* knockdown were derived from cancers of the small and large intestines (highlighted in yellow, Fig. 4A), which had a total of 17 out of 21 cell lines with lower than average shRNA scores (Fig. 4B). Consistent with this finding, significantly higher *DHODH* expression was observed in the Sabetes-Bellver colorectal dataset for adenoma tumor tissues *versus* normal samples (Fig. 4C) (Baldwin et al., 2005; Rhodes et al., 2004; Rhodes et al., 2007; Sabates-Bellver et al., 2007).

Cells overexpressing DHODH may have higher sensitivity to its inhibition. Using data from the Cancer Cell Line Encyclopedia (CCLE), we evaluated mRNA levels of *DHODH* in various cancer cell lines (Fig. 5) (Barretina et al., 2012). The mRNA expression levels of *DHODH* in 1019 cancer cell lines were obtained and converted to z-scores. The top four highest and lowest expressing cell lines are listed in Fig. 5. Frequent overexpression of DHODH mRNA occurs in lung (38% of top 50) and haematopoietic/lymphoid (24% of top 50) tissues. This data correlates well with the Achilles database (Fig. 4). Both lung and haematopoietic/lymphoid are cell lines that frequently possess higher than average cell growth inhibition when exposed to DHODH shRNA. Collectively, this expression data suggests that both lung and haematopoietic/lymphoid cell lines may be more sensitive to DHODH inhibitors than others.

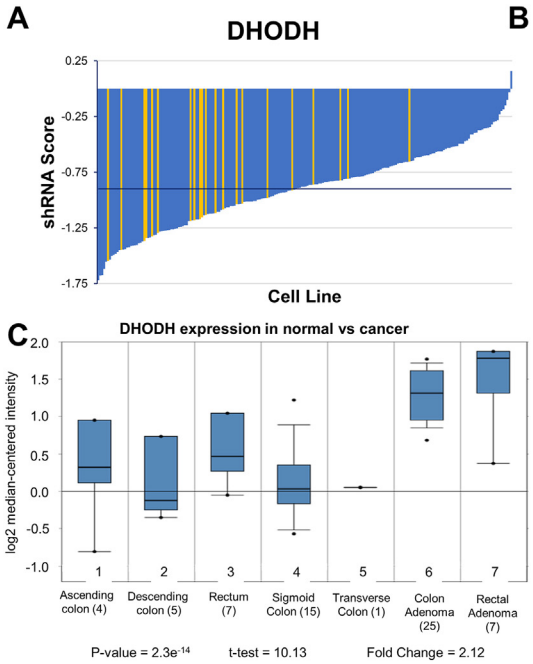
We then evaluated *in vivo DHODH* mRNA expression using data from TCGA (Lee, Palm, Grimes, & Ji, 2015). To perform a pan-disease comparison of *DHODH* expression, log2 TPM expression values were converted to z-scores calculated per patient. Fig. 6 shows that across all 34 diseases (9726 unique patient samples) the median *DHODH* expression is above a z-score of zero indicating that most patients express *DHODH* more than the average of all other genes measured. Among these diseases, liver hepatocellular carcinoma (LIHC) patients tended to express *DHODH* the most (Fig. 6). TCGA disease patient samples were evaluated for reduced survival by comparing survival outcomes for patients with high *DHODH* expression to those with low *DHODH* expression. High *DHODH* expression was associated with reduced survivability in low-grade glioma (LGG) and stomach adenocarcinoma (STAD) patients. High *DHODH* expression was also found to be associated with increased grade in glioma patients from the TCGA in the Rembrandt and Gravendeel studies (Fig. 7) (Gravendeel et al., 2009; Madhavan et al.,

Table 1
DHODH inhibitors evaluated in clinical trials.

Name	Structure	Diseases/Status
Brequinar		Cancer (Dexter et al., 1985) Did not meet objective response in multiple phase II clinical trials for breast (Cody et al., 1993), colon (Dodion et al., 1990), head and neck (Urba et al., 1992), gastrointestinal (Moore et al., 1993), lung (Maroun et al., 1993), melanoma (Natale et al., 1992), and ovarian cancer (Boven et al., 1992) Immunomodulatory (Burris III et al., 1998; Joshi et al., 1997) Used in combination with cyclosporine A, observed to alter PK properties of brequinar (Joshi et al., 1997) Did not gain FDA approval
Leflunomide		FDA-approved for rheumatoid and psoriatic arthritis (Fragoso & Brooks, 2015) Phase I/II clinical trial for multiple myeloma (NCT02509052)
Teriflunomide		FDA approved for multiple sclerosis (Fragoso & Brooks, 2015)
IMU-838		Phase II clinical trial for ulcerative colitis (NCT03341962)
ASLAN003	N-aryl substituted aminoacrylic acid derivative	Phase II clinical trial for AML (NCT03451084)
BAY2402234		Phase I clinical trial for AML (Gradl et al., 2018)
	derivative	

2009; R Core Team, 2018). Within glioma, grade III and IV tumors have higher average mRNA expression of *DHODH* in comparison to normal and grade I tumors. Therefore, in general, higher grade glioma tumors have a higher *DHODH* mRNA expression level.

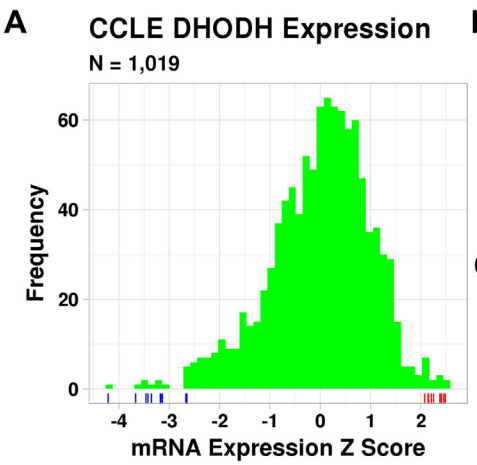
In an effort to determine genes that are correlated with *DHODH* expression in the TCGA patient population, gene set enrichment analysis (GSEA) was used to identify pathways that were enriched with genes that co-express with *DHODH*. Co-expression of *DHODH* in patients was



B

Cancer	shRNA score < -0.897	shRNA score > -0.897
Small/Large intestine	17	4
Kidney	1	9
Breast	5	8
Soft Tissue	1	1
Lung	15	6
Stomach	1	3
Oesophagus	9	1
Haematopoietic/ Lymphoid	17	13
Ovary	15	14
Pancreas	6	11
Skin	2	5
Prostate	1	2
Bone	4	2
CNS	8	27
Endometrium	2	0
Liver	0	1
Urinary Tract	0	3
Pleura	0	2

Fig. 4. DHODH knockdown response from the Achilles Project. A) Plot depicting shRNA scores in 216 cell lines for shRNA targeting *DHODH*. Yellow bars indicate intestinal cancer cell lines. The dark blue horizontal line indicates the mean shRNA score (−0.8972). B) Table depicting the distribution of cell lines with respect to the mean shRNA score. C) DHODH is overexpressed in colon cancer. Box plot was generated using the Sabetes-Bellver colorectal study on the Oncomine platform.



B

Cancer Cell line	Tissue	Z-score
SW620	Large intestine	2.485
MSTO211H	Lung	2.454
KMH2	Haematopoietic and Lymphoid	2.411
HEC50B	Endometrium	2.380

C

Cancer Cell line	Tissue	Z-score
OVMANA	Ovary	-3.423
T173	Bone	-3.463
HS742T	Breast	-3.668
BICR56	Upper Aerodigestive	-4.214

Fig. 5. mRNA levels of DHODH in 1019 cancer cell lines. A) Expression of DHODH in CCLE cell lines, red lines indicate the top ten cell lines with DHODH overexpression and blue lines indicate cell lines with the lowest DHODH expression. B) Top four over- and C) under-expressing cell lines are depicted. Data were generated using the CCLE.

evaluated in LGG, STAD, LIHC, colon adenocarcinoma (COAD), rectum adenocarcinoma (READ), and pancreatic adenocarcinoma (PAAD) using a Pearson correlation measure (Fig. 8) (Subramanian et al., 2005). In each of these patient populations, *DHODH* is co-expressed with genes involved with mitochondrial translation, mitochondrial respiratory complex, the ETC, MYC targets, and translation elongation. These findings are consistent with our above description of DHODH function.

We also performed a correlation analysis independent of annotated gene sets to identify potentially novel genes that are co-expressed with *DHODH*. Using the top 100 *DHODH* co-expressed genes in COAD, LGG, STAD, and PAAD diseases, we identified four common genes across all four diseases (Fig. 9). These genes are DNA polymerase delta 2 (*POLD2*), Tu translation elongation factor, mitochondrial (*TUFM*), peter

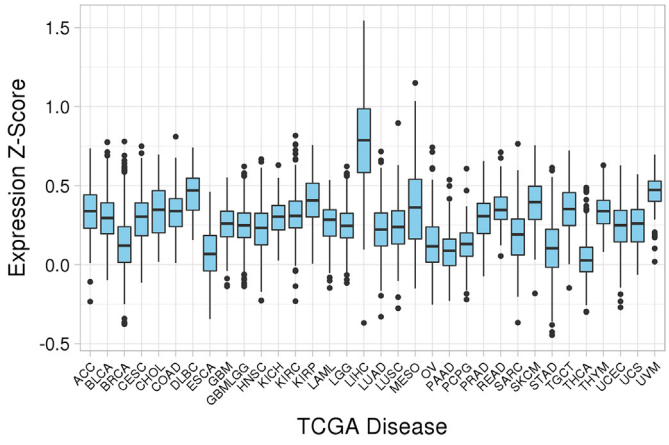


Fig. 6. Pan-disease mRNA expression of DHODH across 34 TCGA diseases. Z-scores were calculated per patient per disease. The majority of patients across all diseases show higher than average expression (z-score = 0) of DHODH. Adrenocortical carcinoma (ACC), bladder urothelial carcinoma (BLCA), breast invasive carcinoma cohort (BRCA), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), lymphoid neoplasm diffuse large B-cell lymphoma (DLBC), esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), glioma (GBMLGG), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal papillary cell carcinoma (KIRP), acute myeloid leukemia (LAML), brain low-grade glioma (LGG), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), mesothelioma (MESO), ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), pheochromocytoma and paraganglioma (PCPG), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ), sarcoma (SARC), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), testicular germ cell tumors (TGCT), thymoma (THYM), uterine corpus endometrial carcinoma (UCEC), uterine carcinosarcoma (UCS), uveal melanoma (UVM).

pan homolog (*PPAN*), and ribosomal subunit RNA processing 9 (*RRP9*). POLD2 is an essential subunit of DNA polymerase delta, a complex that is involved in DNA replication and repair and is one of three major polymerases active in S-phase. DHODH may be connected to POLD2 primarily through providing a sufficient pool of pyrimidine nucleotides. Recent studies have connected DNA repair with *de novo* pyrimidine biosynthesis, although these have been through CAD (Brown, Spinelli, Asara, & Toker, 2017; Givechian, Garner, Garban, Rabizadeh, & Soon-Shiong, 2018). As its name suggests, TUFM participates in protein translation in the mitochondria. During the elongation phase, TUFM forms a complex with GTP and a charged tRNA. Upon correct pairing of the codon:anticodon, GTP is hydrolyzed and TUFM:GDP leaves the ribosome. In the context of cancer, the role of TUFM is not well understood. *TUFM* was identified as a target gene of Ronin, a transcriptional regulator important for embryonic stem cell self-renewal (Dejosez et al., 2010). Interestingly, reversible phosphorylation of the bacterial homolog of TUFM inhibited protein synthesis in quiescent cells, and this phosphorylation site is conserved in the mammalian homolog (Pereira, Gonzalez Jr., & Dworkin, 2015). TUFM has been shown to prevent the epithelial-to-mesenchymal transition and was observed to be successively down-regulated with increases in tumor stage in lung cancer (He et al., 2016). TUFM seems to play a role in differentiation status as does DHODH (see above). However, in colon cancer, TUFM appears to be considerably overexpressed and high expression correlates with poor prognosis (Shi et al., 2012). Additionally, TUFM may play a role in the transition of normal cells to benign and malignant tumors in colorectal tissues (Xi et al., 2017). In ovarian cancer, TUFM was upregulated in cell lines and tumor samples that were partially resistant to standard-of-care chemotherapies and could therefore act as a potential biomarker in predicting sensitivity to treatment (Cruz et al., 2017). PPAN resides in the nucleoli and participates in the maturation of 45S pre-RNA. *PPAN* expression can be stimulated by WNT signaling to trigger ribosomal biogenesis (Bugner, Tecza, Gessert, & Kuhl, 2011; Pfister, Keil, & Kuhl, 2015). Interestingly, high concentrations of leflunomide showed modulation of WNT/β-catenin signaling and was synergistic with an inhibitor of WNT secretion (Chen, Huang, et al., 2016). However, the concentration of leflunomide that was necessary to see these effects was much higher than what is required to see inhibition of DHODH, so it is likely that these observations are a result of an off-target effect of leflunomide (see below). It has been suggested that initiation of ribosomal biogenesis is a result of growth factor stimulation, as it occurs before S-phase entry (Sriskanthadevan-Pirahas, Lee, & Grewal, 2018). Overexpression of mutant RAS caused an increase in the expression of PPAN, which could be reversed by knockdown of MYC (Sriskanthadevan-Pirahas et al., 2018). However, in the presence of a MEK inhibitor and MYC overexpression, rRNA synthesis levels

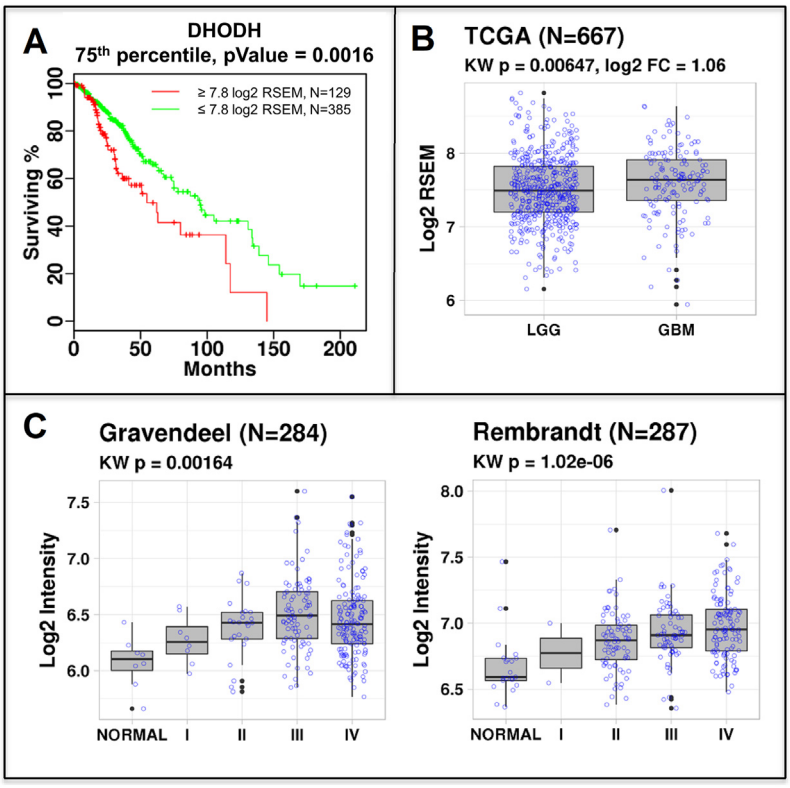


Fig. 7. Analysis of DHODH in glioma. A) Significant reduction in survival is observed for patients with high expression of DHODH in LGG. B) mRNA expression of DHODH is significantly higher in more aggressive GBM gliomas than LGG. C) DHODH expression increases with grade in LGG patients. Kruskal-Wallis and survival analysis statistics were calculated using the R statistical programming language (Gravendeel et al., 2009; Madhavan et al., 2009).

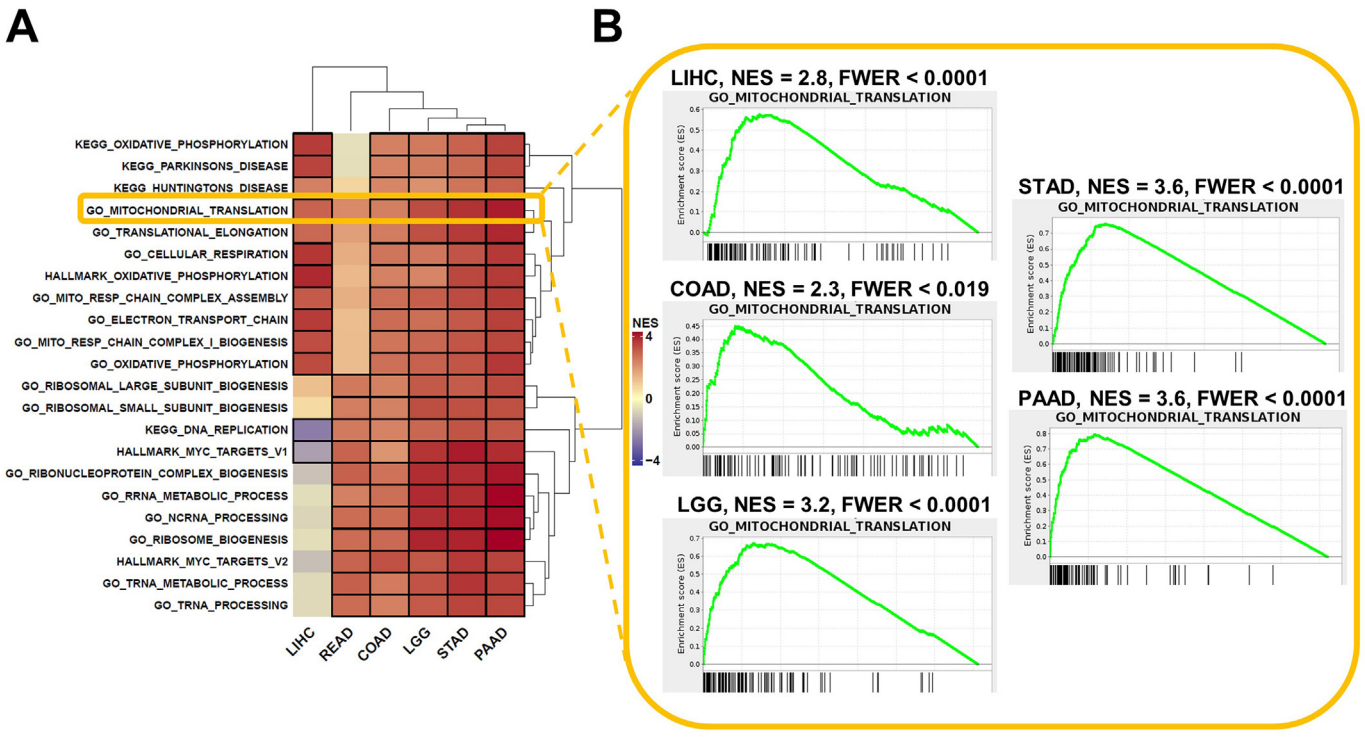


Fig. 8. Common gene sets enriched for genes that are co-expressed with DHODH. A) Gene set enrichment analysis (GSEA) was used to identify enriched pathways with genes that are co-expressed with DHODH. 22 Gene sets were significantly enriched for genes positively correlated with DHODH expression GSEA. Heatmap coloring indicates the normalized enrichment score (NES) and all gene sets with an FWER adjusted p-value <0.05 are colored with a black border. B) GSEA running sum statistic visualizations are shown for the Gene Ontology (GO) category, Mitochondrial Translation was significantly enriched in LIHC, COAD, LGG, STAD, and PAAD TCGA diseases. GSEAv2.2.3 was used with v6 gene sets sourced from MSigDB. 10,000 gene set permutations were performed using weighted mode scoring and Pearson metric (Subramanian et al., 2005). Only genes with evidence of expression in >50% of a disease patient population were considered. Six diseases were evaluated based on association with reduced survivability (LGG, STAD), high expression (LIHC), or experimental evidence (COAD, READ, PAAD).

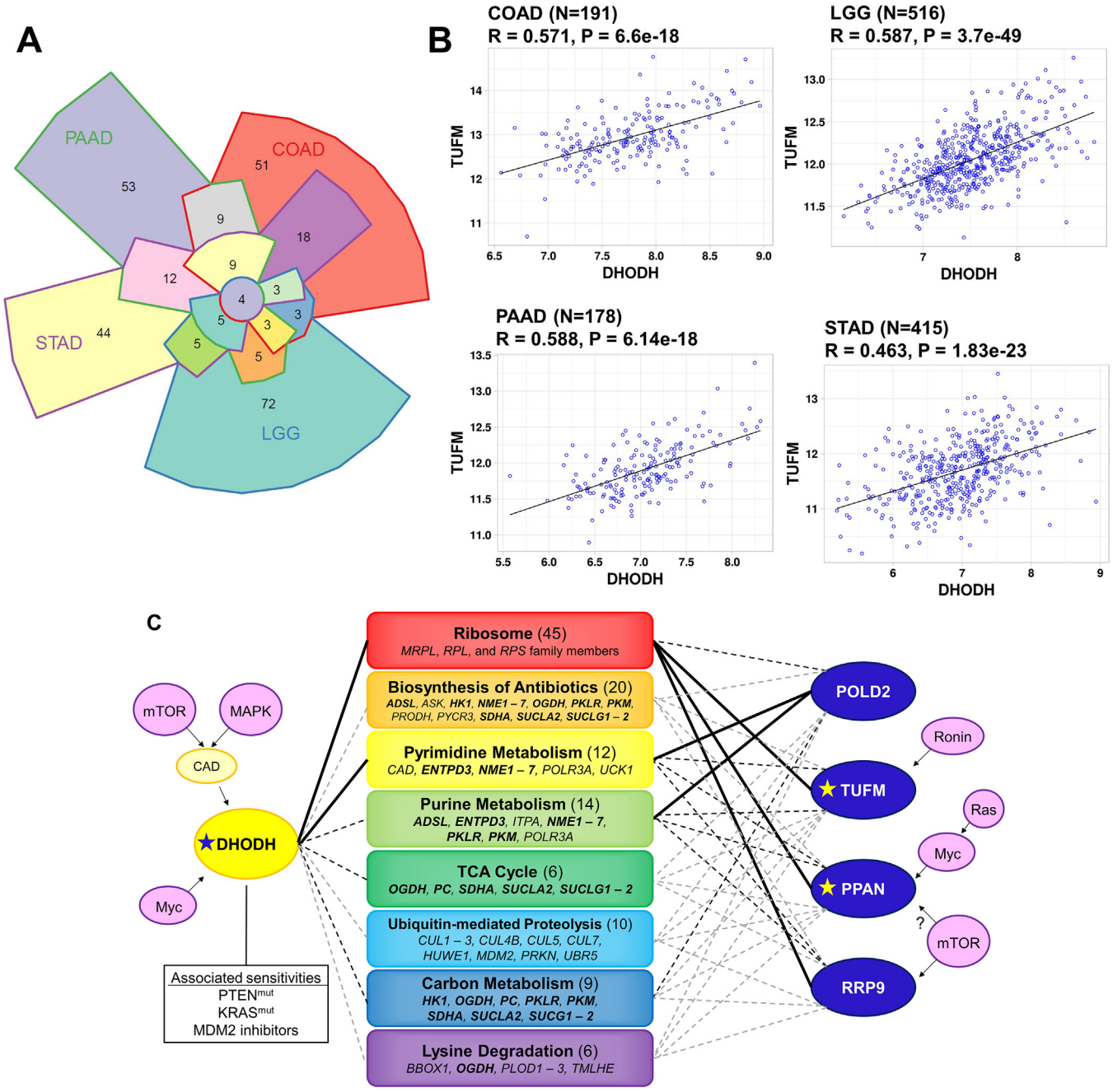


Fig. 9. DHODH gene co-expression was evaluated and compared across multiple TCGA diseases. A) A Chow-Ruskey diagram shows overlap of the top 100 genes correlated with DHODH within COAD, LGG, PAAD, and STAD TCGA diseases. B) TUFM was one of the four top co-expressed genes (POLD2, PPAN, RRP9, TUFM) in common across all four diseases. Gene $\log_2$RSEM expression values are shown in the scatter plots. C) Proposed interactions between DHODH and the four co-expressed genes. These five genes are controlled by similar signaling pathways, which may account for their co-expression. Protein interactions curated in Pathway Commons ultimately identified 200 intermediate genes. Gene set enrichment yielded eight statistically significant KEGG pathways (represented as colored rectangles with rounded edges). Solid line, direct connection; black dashed line, indirect connection; grey dashed line, unknown connection; bolded gene, appears in more than one gene set. Numbers in parentheses indicate the number of genes in the set. Stars indicate proteins that have been implicated with cell differentiation. Gene sets are ranked in the order of significance from top to bottom. The corresponding *p*-values are: ribosome, 8.86E-42; biosynthesis of antibiotics, 1.02E-07; pyrimidine metabolism, 1.21E-05; purine metabolism, 9.3E-05; TCA cycle, 0.00039; ubiquitin-mediated proteolysis, 0.0026; carbon metabolism, 0.0028; lysine degradation, 0.0049. All FDR-adjusted p-values are <0.1.

were not maintained, suggesting that RAS regulates ribosomal biogenesis through additional mechanisms - the mTOR pathway is one such possibility (Ghosh, Rideout, & Grewal, 2014; Sriskanthadevan-Pirahas et al., 2018). PPAN also localizes to the mitochondria where it acts as an anti-apoptotic factor independently of p53 (Pfister et al., 2015). Additionally, PPAN has been shown to be an important component of cellular differentiation for *Xenopus laevis* development (Bugner et al., 2011). Finally, the *RRP9* gene codes for a vital U3 snoRP core protein known as U3-55 k, which is essential for ribosomal RNA synthesis (Chen, Blank, et al., 2016). Ribosomal biogenesis factors are predominantly controlled by S6 kinases, with RRP9 being one of them (Chauvin et al., 2014). S6 kinases are activated through phosphorylation by mTOR, itself a kinase that regulates cell growth and metabolism from a plethora of environmental stimuli (Laplante & Sabatini, 2012). Interestingly, mTOR has been shown to be associated with the histone deacetylase SIRT7, and SIRT7 was recently shown to deacetylate RRP9 (Chen, Blank, et al., 2016; Tsai, Greco, & Cristea, 2014). Both PPAN and RRP9 play a significant role in the regulation of ribosomal RNA synthesis

and may be connected to DHODH through UMP synthesis as well as through upstream oncogenic signaling pathways. Although co-expression does not prove DHODH is directly interacting with these four targets, one might hypothesize a common functional relationship or transcriptional regulation that is worth investigating.

To instantiate such a hypothesis, we constructed a protein interaction network using curated protein interactions sourced from Pathway Commons from KEGG, HPRD, and BioGRID that connected DHODH with the co-expressed genes *POLD2, PPAN, RRP9*, and *TUFM* (Cerami et al., 2011; Chatr-Aryamontri et al., 2017; Goel, Harsha, Pandey, & Prasad, 2012; Kanehisa, Sato, Kawashima, Furumichi, & Tanabe, 2016). Up to three intermediate interactions were allowed between DHODH and the co-expressed genes, generating a network consisting of 621 edges. Gene set enrichment was performed on the 200 intermediate network proteins using the DAVID web service and we identified eight statistically significant KEGG pathways with FDR adjusted p-values <0.1 which are represented as rectangular colored boxes in Fig. 9C (Dennis Jr et al., 2003; Jiao et al., 2012). Consistent with DHODH canonical function, nucleotide metabolism and related metabolic pathways were significantly enriched. Enrichment of the ribosome-related proteins was also consistent with known PPAN and RRP9 involvement in ribosomal biogenesis. In Fig. 9C, we indicate known direct, known indirect, or unknown connections between DHODH, POLD2, TUFM, PPAN, and RRP9 and the eight enriched pathways based on our review of the literature. TUFM participates in the elongation of a nascent peptide and DHODH provides the substrate for production of UMP, a component of rRNA – this known direct connection is represented by a solid black line. Other gene sets are connected through known indirect interactions (indicated by the black dashed lines) to DHODH co-expressed genes according to the literature. For example, POLD2 and the ribosome both require nucleotides to carry out their function. The stars in Fig. 9C indicate proteins that have been linked to differentiation. Finally, our analysis identified connections that have not previously been reported and are potential novel interactions (grey dashed lines).

STRING protein interactions were used to highlight enzymes directly associated with DHODH (Fig. 10) (Szklarczyk et al., 2015). The reported protein-protein associations are primarily within the *de novo* nucleotide synthesis pathways (CAD, carbamoyl phosphate synthetase 1, uridine monophosphate synthetase, and phosphoribosylglycinamide formyltransferase (*de novo* purine)) (Lane & Fan, 2015). Beyond *de novo* pathways, DHODH is reported to associate with the dihydropyrimidinase-like (DPYSL) family. DPYSL is also known as a collapsing response mediator protein (CRMP) family (Tan, Wahdan-Alaswad, Yan, Thiele, & Li, 2013). CRMP1 has been implicated in metastasis and differentiation (F. Tan, Thiele, & Li, 2014) acting as a tumor suppressor in prostate cancer and hindering the epithelial-mesenchymal transition (Cai et al., 2016). Additionally, CRMP may present as a potential cancer biomarker. In neuroblastoma, the activation of MYC selectively decreased the expression of CRMP3, but not CRMP1 or CRMP2. Conversely, siRNA knockdown of *MYC* increased expression of CRMP3, suggesting a unique role for CRMP3 as a biomarker for aggressive neuroblastoma (Gaetano, Matsuo, & Thiele, 1997; Tan et al., 2013). Beyond neuroblastoma, CRMP5 is reported as a biomarker for poor prognosis in osteosarcoma (Wang et al., 2017). However, the complete connection between these proteins, DHODH, and cancer is not fully understood.

Murine knockout studies, performed by the International Mouse Phenotyping Consortium, identified *DHODH* as a homozygous-lethal gene (Brown & Moore, 2012; Meehan et al., 2017) showing that at least one functional allele copy is necessary for survival. However, even heterozygous mutations in *DHODH* may be responsible for health defects, as mutations in *DHODH* were implicated as a cause of Miller syndrome, a genetic disorder characterized by facial and bone abnormalities (Ng et al., 2010). At least 11 different *DHODH* mutations have been identified (Ng et al., 2010). Some amino acid changes in patients

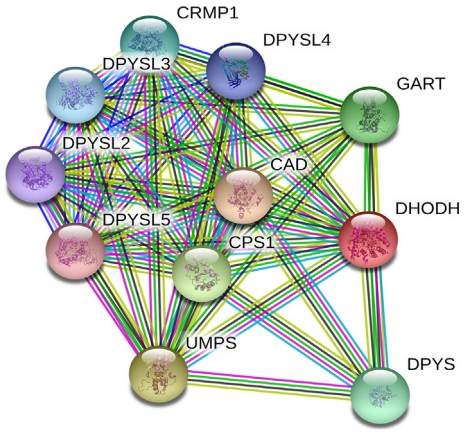


Fig. 10. STRING analysis of protein interactions with DHODH. CAD, carbamoyl phosphate synthetase 2, aspartate transcarbamoylase, and dihydroorotase; CPS1 carbamoyl phosphate synthase 1; CRMP1, collapsing response mediator protein 1; DPYS, dihydropyrimidinase; DPYSL2, dihydropyrimidinase-like 2; DPYSL3, dihydropyrimidinase-like 3; DPYSL4, dihydropyrimidinase-like 4; DPYSL5, dihydropyrimidinase-like 5; GART, phosphoribosylglycinamide formyltransferase; UMPS, uridine monophosphate synthetase.

with Miller's syndrome are R346W and G202A (Ng et al., 2010). These mutations do not have an impact on mitochondrial localization but were later observed to decrease protein stability (Fang et al., 2012). It is unclear how other mutations alter DHODH function in patients with Miller's syndrome. However, there is a clear implication that DHODH function is necessary for normal embryonic development. In fact, the DHODH inhibitor leflunomide is a known teratogen and its use is avoided during pregnancy (Hajdyla-Banas et al., 2009). In murine studies, treatment with leflunomide in pregnant mice caused significant malformations. These malformations did not occur when leflunomide was co-administered with uridine (Fukushima et al., 2007; Fukushima et al., 2009). For embryonic development, this data suggests that DHODH function is necessary to generate nucleotides for continued growth.

Overall, the results of these bioinformatics studies suggest that DHODH is an important oncology target and strengthens the case to pursue drug discovery campaigns aimed at inhibiting DHODH for cancer therapy.

6. Select inhibitors of DHODH

The importance of DHODH in cancer cells has resulted in many drug discovery campaigns. Various DHODH inhibitors belonging to different chemical classes have been reported and previously reviewed (Munier et al., 2013; Vyas & Ghate, 2011). Only select inhibitors targeting human DHODH, which were not previously reviewed, are discussed with the most notable inhibitors, brequinar and leflunomide/teriflunomide, discussed in detail.

6.1. Brequinar

Brequinar is a potent DHODH inhibitor (Table 1, DHODH assay $K_i = 27.4 \pm 1.6$ nM) with significant anticancer properties (Chen et al., 1990). DuPont Pharmaceuticals developed brequinar from a lead compound discovered in the National Cancer Institute's Developmental Therapeutics Program (Dexter et al., 1985). Initial studies with brequinar revealed promising anticancer effects. Dosing at 20–40 mg/kg in murine xenograft models inhibited the growth of breast, lung, stomach, and colon tumors by >90% (Dexter et al., 1985). However, the target was not initially known until a mechanism of action study observed a selective depletion of uridine and cytidine triphosphate (Chen, Ruben, & Dexter, 1986). This

finding ultimately led to the discovery of DHODH as the target. Further studies of brequinar highlighted a schedule dependency, as cells exposed to brequinar for 1–24 h could sustain growth once brequinar was removed. However, when exposed for 48 h, cells were unable to continue growth. Therefore, continual inhibition is necessary to maximize DHODH's anticancer effects, which has been demonstrated in multiple cell lines (Chen et al., 1990; Dexter et al., 1985). Cells with a faster doubling time are more likely to be sensitive to brequinar (Mohamad Fairus et al., 2017). In fact, brequinar showed $IC_{50} < 1$ μM in two breast cancer cell lines (T-47D and MDA-MB-231) with doubling times between 25 and 45 h whereas in slower growing MDA-MB-436 and W3.006, with doubling times of 75–85 h, its IC_{50} was >100 μM (Mohamad Fairus et al., 2017). This data highlights the sensitivity of faster growing cells to DHODH inhibition. These overall impressive results were sufficient to pursue the clinical evaluation of brequinar for the treatment of cancer.

Early clinical evaluation of brequinar gave conflicting results. First, high doses were well tolerated by most patients. Doses at 200–250 mg/m^2 were tolerated with few dose-limiting toxicities (*e.g.*, myelosuppression and nausea) (Arteaga et al., 1989; Bork, Vest, & Hansen, 1989; de Forni et al., 1993; Schwartsmann et al., 1988; Schwartsmann et al., 1989; Schwartsmann et al., 1990). Second, objective responses were not consistently observed in multiple studies leading to the recommendation for increasing the dose in phase II studies (Arteaga et al., 1989; Bork et al., 1989; Braakhuis, van Dongen, Bagnay, van Walsum, & Snow, 1989). However, the results were similar in each study with brequinar failing to produce an objective response in breast (Cody et al., 1993), colon (Dodion et al., 1990), head and neck (Urba et al., 1992), gastrointestinal (Moore et al., 1993), lung (Maroun et al., 1993), melanoma (Natale et al., 1992), and ovarian cancers (Boven et al., 1992). These results were befuddling considering that patient-derived samples of lymphocytes had undetectable DHODH activity for at least a week after stopping treatment with brequinar (Peters et al., 1990). Furthermore, brequinar at 600 mg/m^2 induced plasma uridine depletion (40–80%), which ranged from 6 h to 4 days after dosing. However, a significant rebound of plasma uridine (160–350%) was observed 4–7 days after treatment (Peters et al., 1990). These results suggest that despite potent DHODH inhibition, clinically relevant uridine depletion may not have occurred, possibly due to a sub-optimal dosing regimen as continuous inhibition of DHODH is required to suppress pyrimidine concentrations. With most patients in these trials treated weekly at high doses, it is likely that alternative sources of plasma uridine overcame DHODH inhibition. Hence, brequinar did not meet its objective response, possibly due to poorly chosen dosing regimens.

Recent studies have reported on brequinar's impact beyond cell growth inhibition. Brequinar induced differentiation in AML cells both *in vitro* and *in vivo* suggesting a new utility for this or other DHODH inhibitors (Sykes et al., 2016). Additionally, when dosed in neural crest stem cells, both a brequinar analogue and leflunomide (described below) decreased the cell's self-renewing capabilities. This study suggests that DHODH inhibitors halt neural crest cell growth and limit stem cell renewal (White et al., 2011). Aside from differentiation, brequinar was also observed to overcome tumor necrosis factor-related apoptosis ligand (TRAIL) resistance. Genomic screening was used to identify potential enzymes that may sensitize cells to TRAIL. In this study, siRNA for *DHODH* was found to sensitize resistant cells to TRAIL. Dosing cells with brequinar and TRAIL reproduced these results, which validates DHODH as a viable target for these cells (He et al., 2014). A recent study identified that brequinar was synergistic with prednisone, palbociclib, floxuridine, everolimus, oxaliplatin, and gemcitabine *in vitro* in KRAS mutant cells (Koundinya et al., 2018). Together these studies highlight a future for brequinar beyond single agent use.

6.2. Leflunomide and teriflunomide

Leflunomide is a potent DHODH inhibitor that has gained FDA approval for the treatment of rheumatoid and psoriatic arthritis (Table 1) (Teschner & Burst, 2010). Leflunomide is a prodrug of the active metabolite teriflunomide, itself FDA approved for multiple sclerosis (Oh & O'Connor, 2014). The two are used interchangeably for this indication. Besides arthritis, leflunomide has been evaluated in multiple clinical trials for the treatment of ankylosing syndrome (Haibel, Rudwaleit, Braun, & Sieper, 2005), Crohn's disease (Holtmann, Gerts, Weinman, Galle, & Neurath, 2008; Prajapati et al., 2003), rare autoimmune diseases such as Felty syndrome and granulomatosis with polyangiitis (Sanders & Harisdangkul, 2002), Kimura's disease (Dai et al., 2011), pemphigoid (NCT00802243), organ transplantation (Hardinger et al., 2002; Teschner & Burst, 2010; Williams et al., 2002), sarcoidosis (Bohelay et al., 2014), Still's disease (Pirildar, 2003), systemic lupus (Wu, Xu, Huang, & Wu, 2013), Takayasu arteritis (Unizony, Stone, & Stone, 2013), and uvetitis (Roy, 2007). Despite being marketed primarily for autoimmune disorders, leflunomide also displays anticancer effects. Several studies report anti-proliferative effects in various cell lines, including those derived from multiple myeloma (Baumann et al., 2009), non-small cell lung carcinomas (Jiang, Zhang, Li, Ling, & Jiang, 2017), neuroblastomas (Zhu, Yan, Xiang, Ding, & Cui, 2013), neuroendocrine (Cook et al., 2010), and cancer in medullary thyroids (Alhefdhi, Burke, Redlich, Kunnimalaiyaan, & Chen, 2013). Leflunomide is currently undergoing clinical trials for the treatment of multiple myeloma (NCT02509052). However, the off-target effects of leflunomide therapy complicate attributing these responses to DHODH inhibition. This may be the case in the clinical trial evaluating the combination of leflunomide with vemurafenib in V600 mutant melanoma which had to be terminated early because of adverse events (NCT01611675).

Leflunomide has been reported to target additional enzymes beyond DHODH. One reported off-target effect is due to the aryl hydrocarbon receptor, which may contribute to leflunomide's overall mechanism of action. In fact, melanoma cells were not rescued from leflunomide's antiproliferative effects by uridine supplementation (O'Donnell et al., 2012). DHODH inhibition effects are reversed by uridine supplementation and this result suggests that leflunomide may not be suppressing cell growth solely through DHODH inhibition. Other studies have suggested that leflunomide may inhibit S6 kinase 1, a kinase responsible for ribosomal and CAD phosphorylation (Doscas et al., 2014), but this has not been verified experimentally. Additionally, leflunomide modestly inhibits the EGF-receptor tyrosine kinase at high concentrations ($IC_{50} \approx 40$ μM) (Mattar, Kochhar, Bartlett, Bremer, & Finnegan, 1993). As a result, it is difficult to ascribe leflunomide-induced cellular responses to DHODH alone, due to likely contributions from off-target effects.

Leflunomide also affects other signaling pathways beyond nucleotide biosynthesis. In oral squamous cell carcinoma cells, leflunomide induced upregulation of cyclin A (Ren, Fu, Qiu, & Cui, 2017). Cyclin A is upregulated in S-phase when flux through the *de novo* pyrimidine pathway is abundant and possibly upregulated in response to pyrimidine depletion (Yam, Fung, & Poon, 2002). In murine xenografts of Ehrlich's ascites carcinoma, leflunomide decreased TNF-α and EGF protein levels and this response is associated with immunosuppression (Bahr et al., 2015). Leflunomide has been shown to inhibit *FZD10* gene expression in renal carcinoma cells, whose product is a receptor protein that can initiate WNT/β-catenin signaling (Chen, Huang, et al., 2016). Other cellular studies on leflunomide and teriflunomide have highlighted their chemo-preventative properties for prostate cancer (Hail Jr. et al., 2010). In fact, the effect of leflunomide and teriflunomide in prostate cancer is being evaluated in clinical trials (NCT00004071). However, it is difficult to attribute observed responses to DHODH inhibition, as uridine rescue did not overcome antiproliferative effects in prostate cancer (Hail Jr. et al., 2010). Finally, p53 expression may be a marker of nucleotide depletion as it has been shown that inhibition of *de novo*

pyrimidine biosynthesis resulted in a p53-mediated cell cycle arrest (Khutornenko et al., 2010; Linke, Clarkin, Di Leonardo, Tsou, & Wahl, 1996). Leflunomide and teriflunomide treatment was found to increase the expression of p53, and supplementation of uridine was able to abrogate this effect (Khutornenko et al., 2010; Ladds et al., 2018).

Leflunomide and teriflunomide represent the only FDA approved DHODH inhibitors in clinical use. Therefore, they are commonly chosen for studies on DHODH inhibition. However, there are off-target effects that complicate leflunomide-induced responses and their relationship to DHODH. Nonetheless, the continued use of these inhibitors highlights the potential of long-term DHODH inhibition as a valid clinical strategy.

6.3. Selected human DHODH inhibitors

In general, DHODH inhibitors possess diverse scaffolds (Table 2). A variety of structurally distinct inhibitors of human DHODH have been synthesized and evaluated for potential treatment of cancers (Munier et al., 2013; Vyas & Ghate, 2011), including biphenyl indoles (Bu, Tan, Xing, & Wang, 2017), amides like ML390 (Lewis et al., 2016), derivatives of ascochlorin (Shen et al., 2016), substituted quinolines like brequinar (Batt et al., 1995; Dexter et al., 1985), and leflunomide (O'Donnell et al., 2012; White et al., 2011). There are brequinar-like quinoline-based inhibitors such as FA-613 (Cheung et al., 2017), C44 (Das et al., 2013), compound 11 (Vyas, Variya, & Ghate, 2014), and compound 41 (Madak et al., 2018). Leflunomide-like inhibitors have also been reported, including compound 4 (Sainas et al., 2017). In addition to brequinar and leflunomide-like inhibitors, pyrazole-based analogues 18d (Lucas-Hourani et al., 2015) and 21q (Munier-Lehmann et al., 2015) were reported for human DHODH that had antiviral activity. A tri-substituted benzimidazole inhibitor (compound 8d), which is structurally similar to compound A14 (Li et al., 2016), was reported with an $IC_{50} = 81 \pm 2$ nM (Sitwala et al., 2017). Several of the compounds in Table 2 represent unique classes of DHODH inhibitors without any obvious structural similarity. Despite the multitude of scaffolds, the most reported binding site for these inhibitors is the ubiquinone pocket. Surprisingly, a number of these inhibitors were discovered through cell-based screens and were subsequently validated to target DHODH. This is the same approach used to discover brequinar (Dexter et al., 1985). Inside the ubiquinone pocket, most inhibitors are designed to form an interaction with R136 or form nonpolar interactions with various hydrophobic residues. Common strategies employed for DHODH inhibitor design is well-covered in a recent review (Munier-Lehmann et al., 2013).

6.4. Non-DHODH de novo pyrimidine biosynthesis inhibitors

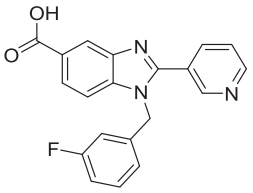
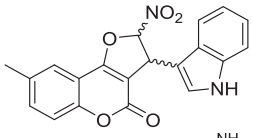
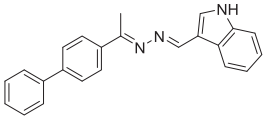
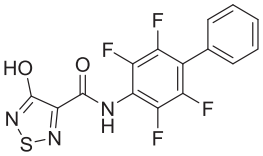
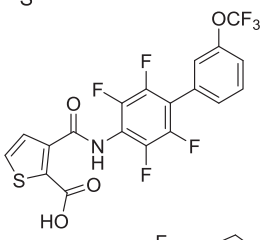
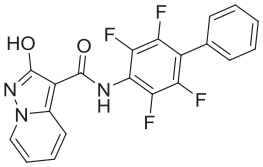
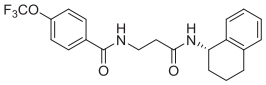
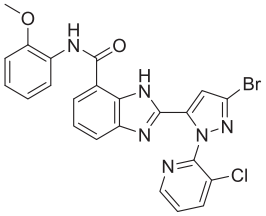
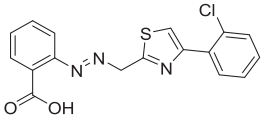
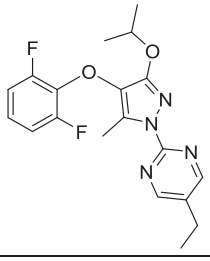
Beyond DHODH, multiple inhibitors of the *de novo* pyrimidine biosynthesis pathway have been evaluated for anticancer activity (Fig. 11). Carbamoyl phosphate synthetase is a reported target for the antitumor agent acivicin (Aoki, Sebolt, & Weber, 1982; Sebolt, Aoki, Eble, Glover, & Weber, 1985). However, acivicin appears to target most enzymes containing a glutamine binding site, thus studies with this compound were not pursued further due to high toxicity (Kreuzer, Bach, Forler, & Sieber, 2015). Aspartate carbamoyltransferase has been targeted by N-(phosphonacetyl)-L-aspartate (PALA), a potent inhibitor ($K_i = 26$ nM) for the murine enzyme (Christopherson, Lyons, & Wilson, 2002; Collins & Stark, 1971). PALA performed well against solid murine tumors, but did not fare well in clinical trials (Christopherson et al., 2002; Grem, King, O'Dwyer, & Leyland-Jones, 1988). In humans, PALA caused a meager decrease in plasma uridine concentrations and only one patient was a reported responder (Grem et al., 1988; Karle, Anderson, Erlichman, & Cysyk, 1980). A metabolic resistance mechanism was proposed in which PALA-induced inhibition of aspartate carbamoyltransferase led to a significant increase in carbamoyl phosphate, the substrate. Sufficient concentrations may have been reached to displace PALA and continue flux through the *de novo* pathway (Christopherson et al., 2002; Christopherson & Duggleby, 1983). Efforts have been made to target dihydroorotase to treat cancer, the most notable being 4-mecapto-6-oxo-1,4-azaphosphinane-2-carboxylic acid 4-oxide (MOAC). However, these inhibitors have shown minimal anticancer activity (Adams, Meek, Mong, Johnson, & Metcalf, 1988; Christopherson et al., 2002; Manthey, Huang, Bubb, & Christopherson, 1998). Additionally, attempts have been made to target OMP decarboxylase by pyrazofurin, which is phosphorylated in the cell to generate an active OMP decarboxylase inhibitor (Dix, Lehman, Jakubowski, Moyer, & Handschumacher, 1979). In clinical trials, pyrazofurin did not fare well as limited objective responses were observed (Cadman, Dix, & Handschumacher, 1978; Gutowski et al., 1975; Ohnuma, Roboz, Shapiro, & Holland, 1977).

7. Combination approaches to increase efficacy of DHODH inhibitors

Results from the multitude of *de novo* pyrimidine inhibitors in anticancer clinical trials depict a mixed outlook for single agent DHODH inhibition. As previously discussed, brequinar produced minimal objective responses in clinical trials. Results were similar for all inhibitors of the *de novo* pyrimidine biosynthesis pathway evaluated in the clinic (*e.g.*, PALA and pyrazofurin). However, a significant problem in targeting *de novo* pyrimidine biosynthesis is that cells may alternatively source nucleotides from the salvage pathway. Results from uridine rescue experiments have led to the hypothesis that a DHODH inhibitor may require concomitant administration of other agents to be successful (Peters, Sharma, Laurensse, & Pinedo, 1987; Schwartsmann et al., 1988).

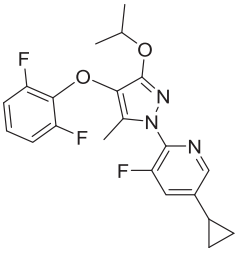
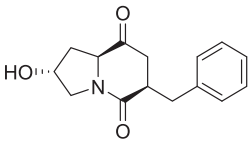
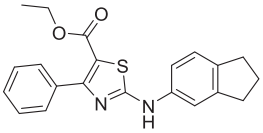
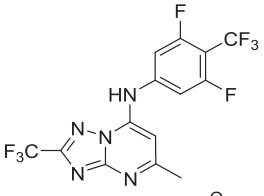
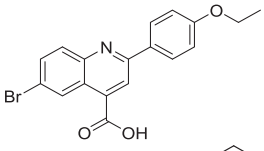
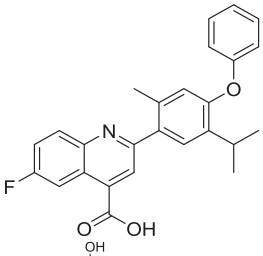
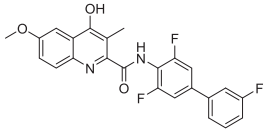
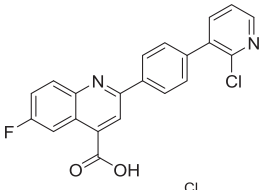
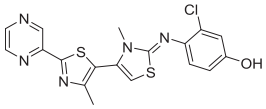
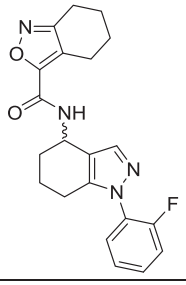
The combination of brequinar and 5-fluorouracil (5-FU) has been evaluated to assess whether brequinar-induced uridine depletion would improve 5-FU activity. In cells, 5-FU is metabolized into FdUMP and inhibits thymidylate synthetase, which catalyzes the formation of thymidine monophosphate from deoxyuridine monophosphate (Longley, Harkin, & Johnston, 2003; Wilson, Danenberg, Johnston, Lenz, & Ladner, 2014). A combination of brequinar and 5-FU was proposed to increase generation of FdUMP by decreasing intracellular nucleotide concentrations. Initial evaluation of this combination utilized a low dose of brequinar to assess whether pretreatment would improve the activity of 5-FU or 5-FU with leucovorin. The results indicated that pretreatment with brequinar, 24 or 48 h in advance of 5-FU dosing, improves potency and that brequinar pretreated cells had significantly higher concentrations of FdUMP compared to cells treated with 5-FU alone (Chen & Erlichman, 1992). However, the addition of leucovorin to this combination diminishes the effectiveness as brequinar pretreatment did not significantly improve the 5-FU and leucovorin combination (Chen & Erlichman, 1992). A larger study evaluating 5-FU and brequinar *in vivo* suggested the combination provided a synergistic effect in colon cancer cells at low uridine concentrations. However, at higher concentrations, this combination did not perform well. Studies were also conducted in colon 26 (estimated intracellular [uridine] of 10 μM) and colon 38 (estimated intracellular [uridine] of 50–100 μM) cell lines (Peters et al., 1987; Peters, Kraal, & Pinedo, 1992). When applied *in vivo*, the 5-FU + brequinar combination significantly decreased the colon 26 tumor weight, but did not significantly affect the colon 28 tumor compared to 5-FU alone (Peters et al., 1992). A third compound, dipyridamole (DPM), was then added to improve the combination *in vitro*. DPM is an equilibrative nucleoside transport inhibitor believed to hinder nucleobase/nucleoside transport across the cell membrane. This three inhibitor cocktail combination reduced cell growth at low concentrations of uridine, but was unable to inhibit cell growth in the presence of 50 μM uridine (Peters et al., 1992). Further *in vivo* studies on the combination demonstrated a significant decrease in tumor weight when comparing brequinar as a single agent to brequinar plus 5-FU

Table 2
Selected recently published DHODH inhibitors.

Name	Structure	Predicted Binding Site	Diseases/Status
Compound **8d**		Ubiquinone pocket	DHODH IC_{50} = 81 ± 2 nM (Sitwala et al., 2017)
DD778			Racemate* Antiviral (Lucas-Hourani et al., 2017)
BEHI			Anticancer cell data (Bu et al., 2017) A549 IC_{50} = 20.5 μM $4T_1$ IC_{50} = 18.5 μM
Compound **4**		Ubiquinone pocket	Antiproliferative DHODH IC_{50} = 0.0016 ± 0.001 μM Jurkat IC_{50} = 1.04 ± 0.04 μM (Sainas et al., 2017)
Compound **1**		Ubiquinone pocket	DHODH IC_{50} = 1.5 ± 0.2 nM (Marschall et al., 2013)
Compound **4**		Ubiquinone pocket	DHODH IC_{50} = 0.0012 ± 0.0002 μM (Sainas et al., 2018)
ML390		Ubiquinone pocket	Antiproliferative DHODH IC_{50} = 0.56 ± 0.1 μM EC_{50} = 1.8 ± 0.6 μM for ER-HOX-GFP cells (Lewis et al., 2016)
Compound **A14**			DHODH IC_{50} = 0.178 ± 0.065 μM (Li et al., 2016)
Compound **19**		Ubiquinone pocket	DHODH IC_{50} = 0.0322 ± 0.0002 μM (Li et al., 2015)
Compound **18d**			DHODH IC_{50} = 25 ± 5 nM Jurkat IC_{50} = 0.02 μM (Lucas-Hourani et al., 2015)

(continued on next page)

Table 2 (*continued*)

Name	Structure	Predicted Binding Site	Diseases/Status
Compound **21q**			DHODH IC_{50} = 13 nM (Munier-Lehmann et al., 2015)
Fr. 1-4		Ubiquinone pocket	DHODH IC_{50} = 0.773 ± 0.007 μM (Jiang et al., 2015)
Compound **44**		Ubiquinone pocket	DHODH IC_{50} = 0.026 ± 0.005 μM (Zhu et al., 2015)
DSM338		Ubiquinone pocket	DHODH IC_{50} = 1.6 μM (Deng et al., 2014)
FA-613			Antiviral suggested as DHODH inhibitor (Cheung et al., 2017)
C44		Ubiquinone pocket	DHODH IC_{50} = 1 nM (Das et al., 2013)
Compound **11**			DHODH IC_{50} = 0.94 ± 0.06 μM A-375 IC_{50} = 5.03 μM (Vyas et al., 2014)
Compound **41**		Ubiquinone pocket	DHODH IC_{50} = 9.71 ± 1.4 nM (Madak et al., 2018)
Compound **2**			DHODH IC_{50} = ~10 nM (Koundinya et al., 2018)
HZ05		Ubiquinone pocket	*(R)* DHODH IC_{50} = 11 ± 0.94 nM *(S)* DHODH IC_{50} = 6.7 ± 0.67 μM (Ladds et al., 2018)

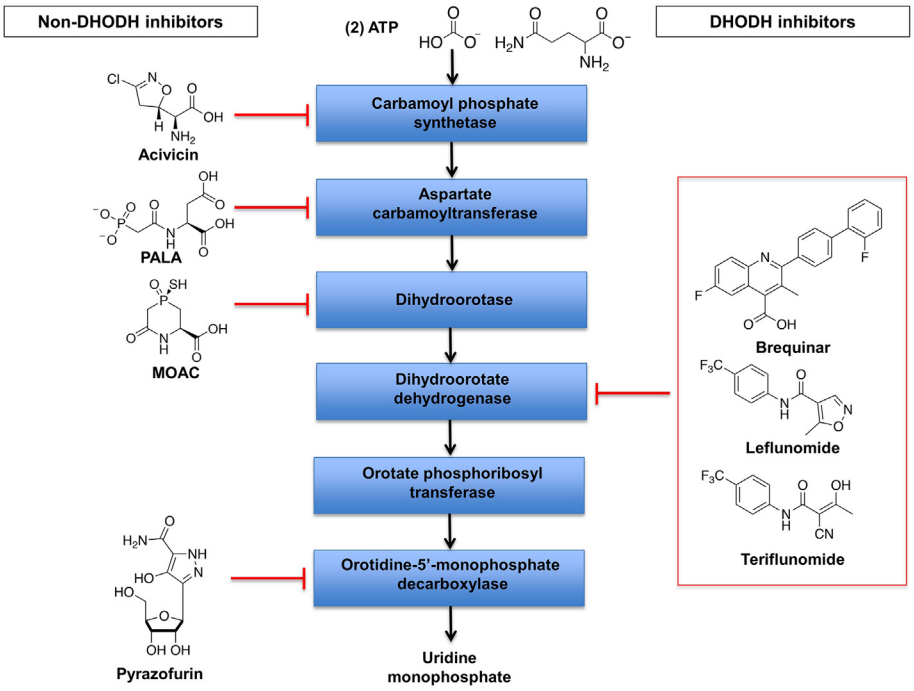


Fig. 11. Selected inhibitors of the *de novo* pyrimidine biosynthesis pathway.

(Pizzorno, Wiegand, Lentz, & Handschumacher, 1992). The combination was tested in a Phase I study, in which weekly doses as high as 600 mg/m^2 brequinar and 600 mg/m^2 of 5-FU were administered. However, no objective responses were observed in 25 patients with refractory solid tumors (Buzaid et al., 1995). Brequinar doses as high as 400 mg/m^2 were observed to decrease baseline uridine levels by >50% (Buzaid et al., 1995). Beyond combinations with 5-FU, brequinar has also been evaluated in combination with cisplatin. Unfortunately, the results in a phase I study were similar with no objective responses (Burris III et al., 1998).

Other combination therapy studies have been pursued with non-DHODH inhibitors of *de novo* biosynthesis. 5-FU and DPM have been evaluated in combinations with other *de novo* pyrimidine biosynthesis inhibitors. PALA and 5-FU were evaluated in multiple anticancer clinical trials, but the combination showed little advantage in comparison to 5-FU alone (Ardalan, Jamin, Jayaram, & Presant, 1984; Weiss, Ervin, Meshad, & Kufe, 1982). The combination of PALA, 5-FU, and leucovorin did not perform much better in a clinical trial for pancreatic ductal adenocarcinoma. From a total of 26 patients, only 3 showed partial responses to combination therapy (12%) (Whitehead et al., 2004). Beyond 5-FU, PALA was investigated with DPM. This combination progressed to a phase I clinical trial, but only 4 of 65 patients responded to the therapy (Markman, Chan, Cleary, & Howell, 1987). An additional phase II study evaluating PALA and DPM for soft tissue sarcoma concluded that the combination was not better than PALA alone, which had previously failed to induce objective responses in clinical trials (Casper et al., 1991). Aside from PALA, a combination of acivicin and DPM has been evaluated in patients diagnosed with diverse tumor types. However, this did not reach objective responses despite achieving plasma concentrations of DPM that were sufficient to inhibit nucleoside transport *in vitro* (Fischer, Pamukcu, Bittner, & Willson, 1984; Willson et al., 1988).

The results from these clinical studies raise an important question. Why have inhibitors of pyrimidine biosynthesis, and in particular DHODH, been unsuccessful in clinical trials? As mentioned above, the salvage pathway may provide a rationale.

Extracellular uridine can be transported across the cell membrane to enter the nucleotide salvage pathway. A colon cancer cell line supplemented with uridine was capable of growing normally in the presence of 1 μM brequinar (Peters et al., 1992). The supplemented uridine may be transported into the cell by the solute carrier 28 family (concentrative nucleoside transporters, CNT1–3), solute carrier 29 family (equilibrative nucleoside transporter, ENT1–4), or select members of the solute carrier 35 family (SLC35) (Fig. 12) (Song, 2013; Young, Yao, Baldwin, Cass, & Baldwin, 2013). Once inside the cell, uridine can be converted to UMP by uridine kinase, bypassing pyrimidine depletion induced by DHODH inhibition (Greenberg, Schumm, & Webb, 1977). Thus, an extensive network of salvage enzymes and transporters can generate the required nucleotides from extracellular sources of nucleobases/nucleosides to sustain cell growth. However, it is unclear why combinations of DHODH inhibitors, such as brequinar with DPM, have been unsuccessful. A potential reason why this did not produce significant efficacy is that this combination may be cytostatic, with cancer cells regaining the ability to grow when the drugs are removed. We predict that the combination of DHODH inhibition with cytotoxic chemotherapy or radiation is likely to be cytotoxic rather than cytostatic and may improve efficacy. DPM halts the flux of nucleobases/nucleosides across the cell membrane by targeting the ENT isoforms (Young et al., 2013). While other nucleoside transporters are present, the ENT family is thought to be the predominant source of uridine flux across the membrane as most ENT isoforms catalyze facilitative diffusion and have a higher turnover rate in comparison to CNTs (ENT1: 200 uridine molecules/s; CNT1: 10 uridine molecules/s) (Smith et al., 2004; Young et al., 2013). Despite the slower rate of transport, it is possible that the sodium- or proton-coupled CNTs sustain the required intracellular nucleotide concentrations (Young et al., 2013). The role of nucleoside transporters from the SLC35 family is unknown. Select members of the large family transport UDP analogues across membranes. For example, the SLC35 family member UGT catalyzes the transport of UDP-galactose and UDP-*N*-acetylgalactosamine across the membrane, but requires UMP as an antiport exchange substrate (Song, 2013). It is unclear if these transporters would be active in cells with low UMP concentrations. For successful combination therapy, inhibitors of both CNT and ENT administered with DHODH inhibitors might be necessary.

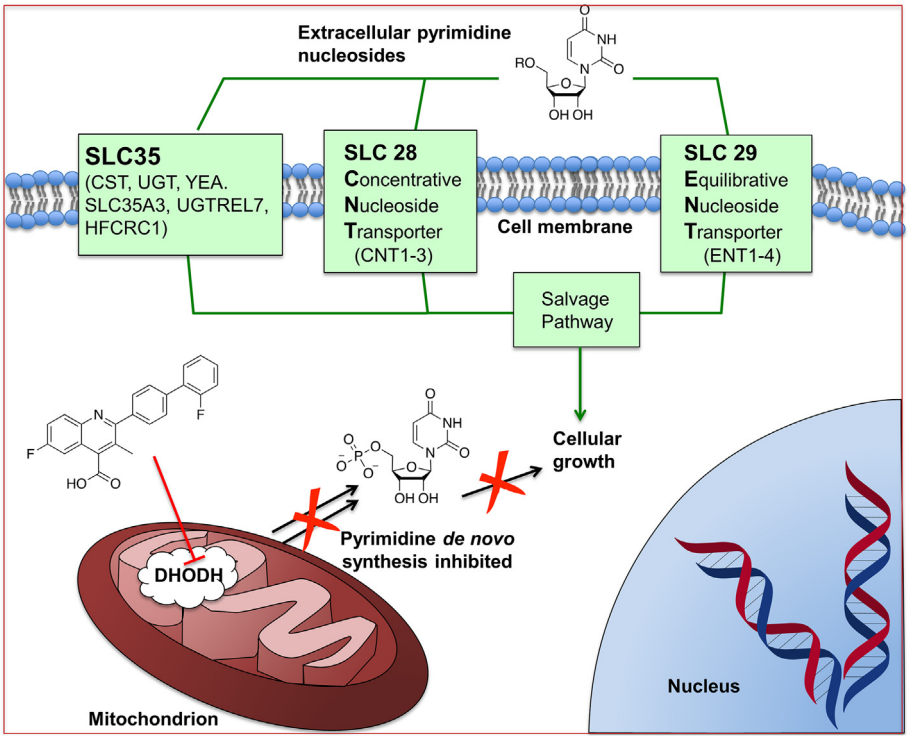


Fig. 12. Proposed compensatory resistance mechanism to DHODH inhibition. Several transporters participate in the salvage pathway and provide an alternative source of nucleosides to the cell when *de novo* pyrimidine biosynthesis is inhibited.

8. Future of DHODH-targeted therapy

We believe the future of DHODH-targeted therapy in cancer lies in multi-drug combination treatments to produce *in vivo* synergy. Despite the setbacks of brequinar in clinical trials, DHODH remains a viable anticancer target. The pyrimidine depletion induced by DHODH inhibition may sensitize cells to better outcomes with current chemotherapy options. In fact, several studies have previously implicated DHODH inhibition as key to overcoming chemotherapy resistance (Brown et al., 2017; He et al., 2014; Shukla et al., 2017).

DHODH inhibition sensitizes cancer cells to conventional chemotherapy and overcomes resistance mechanisms by targeting metabolic dependencies. For example, leflunomide is key to overcoming chemotherapy resistance in triple-negative breast cancer cell lines (Brown et al., 2017). Triple-negative breast cancer cell lines exposed to genotoxic agents increased flux through the *de novo* pyrimidine pathway, resulting in increased nucleotide concentrations to facilitate DNA repair and decreasing their sensitivity to genotoxic agents. Pretreatment with leflunomide induced pyrimidine depletion in triple-negative breast cancer cells and overcame doxorubicin resistance (Brown et al., 2017). Additionally, as previously described, brequinar increased cell sensitivity to TRAIL therapy (He et al., 2014). Beyond doxorubicin and TRAIL, DHODH inhibitors have been shown to sensitize cells to DNA substrate mimics. When leflunomide and gemcitabine were used in combination, the effect was more significant than with single-agent dosing. Similar to combinations of brequinar and 5-FU, leflunomide-induced pyrimidine depletion may have led to a higher incorporation of gemcitabine (Shukla et al., 2017). Similar combinations, such as teriflunomide with 5-azacytidine, were evaluated in resistant cell lines in 5-azacytidine-resistant leukemic cells (Imanishi et al., 2017), and leflunomide with fludarabine in fludarabine-resistant chronic lymphocytic leukemia cells (Dietrich et al., 2012). It is unclear if these combinations would have a similar effect clinically as brequinar and 5-FU. However, preclinical data suggests that DHODH-induced pyrimidine depletion may be used to overcome certain acquired resistance mechanisms.

With the recent expanse of technology to evaluate synthetic lethality, new combinations with DHODH inhibitors may be uncovered (O'Neil, Bailey, & Hieter, 2017). Many oncogenic backgrounds share synthetic lethality with DHODH. Cells with mutant PTEN have increased glutamine metabolism and are sensitive to DHODH inhibition (Mathur et al., 2017). Teriflunomide was synergistic with the BRAF(V600E) inhibitor PLX4720 in melanoma cells (White et al., 2011). KRAS mutant cells were found to be more sensitive to DHODH inhibition over KRAS WT cells (Koundinya et al., 2018). Interestingly, DHODH inhibitors were found to increase p53 synthesis and were synergistic with an MDM2 inhibitor (Ladds et al., 2018). While more studies are needed, these results suggest that targeting pyrimidine biosynthesis with DHODH inhibitors may be a pivotal point of exploitation in specific genomic backgrounds.

Future DHODH-targeted therapy may be improved by identifying patient populations that will be responsive to DHODH inhibition. The recent decrease in the cost of genomic profiling makes effective personalized medicine increasingly feasible and may be used to develop and test biomarkers that predict DHODH sensitivity. Several biomarkers have been identified, including mTORC1. In fludarabine-resistant cells, mTORC1 is overexpressed, and these cells were surprisingly sensitive to both leflunomide and other inhibitors of the *de novo* pyrimidine pathway (PALA) (Sharma et al., 2014). A similar response was observed with inhibitors of *de novo* purine biosynthesis suggesting a potential dependence of cells expressing mTORC1 on *de novo* nucleotide biosynthesis pathways (Valvezan et al., 2017). In addition to mTORC1, PTEN may also be a valuable biomarker. Cell lines with mutant PTEN, a known hallmark of resistant prostate and breast cancer, were remarkably sensitive to both brequinar and leflunomide (Keniry & Parsons, 2008; Mathur et al., 2017). While more data are needed, DHODH-targeted therapy will be more effective if validated biomarkers are available to predict responsive patient populations.

DHODH-targeted therapy may also be better suited for non-solid tumors. Previous clinical trials focused primarily on solid tumors and showed minimal objective responses. Brequinar, for example, was never evaluated clinically in AML patients. As myelosuppression was a

common side effect of brequinar therapy, DHODH inhibition may be more efficacious in patients with leukemia (de Forni et al., 1993; Schwartsmann et al., 1990). In fact, the extent of myelosuppression observed during clinical trials was enough to consider brequinar as a potential immunosuppressant (Chastanet et al., 1998; D'Silva et al., 1996; Makowka, Sher, & Cramer, 1993; Wang, Qu, Stepkowski, Chou, & Kahan, 1996). DHODH inhibition is known to suppress the immune system as two DHODH inhibitors, leflunomide and teriflunomide, are FDA approved for autoimmune diseases, including rheumatoid arthritis (Sanders & Harisdangkul, 2002). Therefore, DHODH-targeted therapy may be more effective in leukemia, and current clinical trials are investigating this hypothesis.

9. Conclusions

DHODH is a promising anticancer target that alters cellular nucleotide concentrations. Inhibition of DHODH is pharmacologically relevant in several diseases, including cancer. DHODH inhibition induces pyrimidine depletion and halts cell cycle progression in S-phase. Despite this, brequinar failed to achieve an objective response in multiple cancer clinical trials. Our bioinformatics analysis provides convincing data that DHODH remains a viable anti-cancer target. Rather than single agent dosing, the future of DHODH inhibitors may lie in appropriate combination therapy that sensitizes treatment of common chemotherapy agents by overcoming resistance mechanisms. Additionally, DHODH inhibition induced differentiation in AML and may be used to target cancer stem cells. Collectively, there is considerable optimism surrounding DHODH-targeted therapy for cancer.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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