Article:
Ramos, L. orcid.org/0000-0002-3808-6853, Henriksson, M. orcid.org/0000-0002-2230-4887, Helleday, T. orcid.org/0000-0002-7384-092X et al. (1 more author) (2024) Targeting MTHFD2 to exploit cancer-specific metabolism and the DNA damage response. Cancer Research, 84 (1). pp. 9-16. ISSN 0008-5472

https://doi.org/10.1158/0008-5472.can-23-1290

Targeting MTHFD2 to exploit cancer-specific metabolism and the DNA damage response

Running Title: Targeting MTHFD2 in cancer

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Conflict of Interest

LR and ACG declare no conflicts of interest. MTHFD1/2 inhibitors are developed toward the clinic by the company One-Carbon Therapeutics AB. T.H. owns shares in One-Carbon Therapeutics AB and M.H. has rights to One-Carbon Therapeutics AB.

Abstract

The one-carbon folate enzyme methylenetetrahydrofolate dehydrogenase/cyclohydrolase 2 (MTHFD2) is a promising therapeutic target in cancer. MTHFD2 is upregulated across numerous cancer types, promotes growth and metastasis of cancer, and correlates with poorer survival. Recent studies have developed small molecule inhibitors to the isozymes MTHFD2 and MTHFD1 that show promise as anti-cancer agents through different mechanisms. This review discusses the current understanding of the function of MTHFD2 in cancer and the status of inhibitors for treating MTHFD2-overexpressing cancers.

Introduction

Cellular metabolism is upregulated in cancer cells to support tumour growth and metastasis. A key part of cancer metabolism is the one-carbon (1C) folate cycle, which supports nucleotide and amino acid synthesis that are required to sustain rapid proliferation. Targeting one-carbon metabolism for cancer treatment dates as far back as 1948 with the use of antifolates by Sydney Farber to treat leukemia (1). Early anti-folate chemotherapies, such as methotrexate, remain effective cancer treatments today, but side effects are common and can be severe (2,3).

Methylenetetrahydrofolate dehydrogenase/cyclohydrolase 2 (MTHFD2) as a cancer target, has been in the spotlight, gaining attention since 2012 when Jain and co-workers demonstrated MTHFD2 being one of the most highly overexpressed metabolic enzymes in cancer (4). This was supported by a metanalysis by Nilsson *et al.,* identifying that MTHFD2 is upregulated across 16 cancer types (5). This has led to numerous studies showing MTHFD2 knockdown impairs cancer growth, and importantly, the development of inhibitors targeting MTHFD2-expressing cancers that are effective in mouse models (6-8).

One-Carbon Metabolism Overview

One-carbon metabolism involves the production of the universal one-carbon acceptor tetrahydrofolate (THF) from dietary folate, and the catabolism of serine for the donation of one-carbon units (**Fig. 1**). The pathway is integral for thymidine and purine biosynthesis, maintenance of amino acids (serine, glycine and methionine) as well as redox homeostasis. Global knockout of *Mthfd1, Mthfd1l* or *Mthfd2* in mice is embryonic lethal, demonstrating the importance of 1C metabolism in early embryonic development (9-11). Interestingly, genome-wide CRISPR-Cas9 screening data suggest that loss of *MTHFD2* or *MTHFD1L* appears not to significantly reduce viability in the majority of cell lines, while loss of *MTHFD1* is required for survival of a subset of cell lines (12).

The 1C cycle is compartmentalised between the mitochondria, cytosol and nucleus through localised expression of serine hydroxymethyltransferase (SHMT) and MTHFD enzymes (**Fig. 1**) (13-15). The enzymes involved in the 1C cycle between the mitochondria and cytosol/nucleus overlap in the reversible reactions they facilitate (13,16). In the cytosol, the trifunctional enzyme MTHFD1 interconverts formate, 10-formyl-THF, 5,10-methenyl-THF and 5,10-methylene-THF. In the mitochondria, these same reactions are performed by the formyl-THF synthetase MTHFD1L and the bifunctional dehydrogenase/cyclohydrolase enzymes MTHFD2 or MTHFD2L (**Fig. 1**). Interconversion of THF and 5,10-methylene-THF is performed by SHMT1 in the cytosol and SHMT2 in the mitochondria. The mitochondrial and cytosolic pathways are connected by the transfer of serine/glycine and formate between the compartments, whereas the 1C-loaded metabolites have not been shown to cross the mitochondrial membrane. The reason for the compartmentalisation has been proposed by Ducker and Rabinowitz to allow cells to separate glycolysis from 1C metabolism by maintaining redox homeostasis in the different cellular compartments (13).

The demands of different cellular compartments control the flow of the 1C cycle, allowing cells to regulate metabolite homeostasis and sustain proliferation and development of normal tissues. The

flow direction can be measured through isotope tracing experiments using [2,3,3-^{2}H]serine. [2,3,3-^{2}H]serine contains three ^{2}H in positions 2, 3, 3 (16,17). The incorporation of ^{2}H into metabolites (e.g. dTMP) can then be measured by LC-MS or NMR to identify if they were produced via the cytosolic or mitochondrial 1C pathway. Ducker *et al.* found that in standard culture conditions most cancer cells produce formate via the mitochondria, supported by studies from Meiser *et al*. showing mitochondrial formate overflow is a hallmark of cancer (18,19). The loss of mitochondrial one-carbon metabolism (e.g. via *Mthfd2* knockdown) reverses one-carbon flux and subsequently cells become reliant on SHMT1 for synthesis of thymidine and purines (16). This contrasts with cells cultured under physiological folate levels (200nM, compared to 2.2 µM in RPMI), where many cell lines produce one-carbon units via the cytosolic pathway (20). At this stage, it is unclear how data from *in vitro* isotope tracing studies translates to 1C flux *in vivo*, as there is only limited data from mouse models. But, it is clear that culture conditions have a significant impact on 1C flux and should be carefully considered when studying the biology of 1C metabolism and development of therapeutics targeting 1C metabolism. Moreover, this extends to animal models where metabolite levels vary across species, including thymidine which is 100-fold higher and hypoxanthine which is 100-1000-fold lower in mice than humans (21-23). As such, using mouse tumour models to test drugs targeting 1C enzymes needs to be approached with caution.

One-carbon enzymes are also expressed in the nucleus, including MTHFD1, SHMT1, MTHFD2 and SHMT2a (SHMT2 isoform). Nuclear 1C enzymes produce 5,10-methylene-THF, allowing *de novo* synthesis of thymidine by thymidylate synthase (TYMS) which converts dUMP to dTMP (9,24). Production of thymidine is essential for DNA synthesis and repair, and helps to maintain genomic stability.

The importance of the MTHFD enzymes in normal growth and development make the upregulation of these enzymes valuable to cancer cells as well. MTHFD1 and MTHFD1L were found to be an important predictor of prognosis in hepatocellular carcinoma and bladder cancer, respectively (25,26). MTHFD2 is normally active during embryogenesis, and plays a role in proliferation and production of inflammatory cytokines during T cell activation (27) . In most adult tissues, MTHFD2 expression is low or absent, and genetic profiling of cancer versus normal cells revealed MTHFD2 is upregulated in cancer (5,11). Thus, MTHFD enzymes demonstrate a significant role in normal development, and their dysregulation has important implications in cancer.

MTHFD2 Significance in Cancer

In cancer, the upregulation of mitochondrial 1C metabolism leads to an over production of formate or 'formate overflow' from the mitochondria (18,19,28,29). This formate can then be used by cytosolic 1C enzymes to fuel nucleotide synthesis, which is in higher demand in cancer cells. There is surmounting evidence that formate overflow is driven by upregulation of MTHFD2 (18,19,28,29).

In a meta-analysis of 20,103 metabolic genes expressed in cancer, *MTHFD2* was one of the top three overexpressed genes in 19 different tumour types (5). Analysis of MTHFD2 expression via The Cancer Genome Atlas project, found 25 out of 31 human cancer cell lines had overexpression of MTHFD2 (30).

Overexpression of the MTHFD2 protein similarly demonstrated significant representation in 16 tumour types from 176 patient samples (5).

Mouse models have demonstrated the biological importance of MTHFD2 in tumour development and metastasis across many different cancer types (**Table 1**). Short hairpin RNA knockdown of MTHFD2 in cancer cell lines and patient derived cells impairs tumour growth in acute myeloid leukaemia (AML) (8,31) and colorectal cancer (6) *in vitro* and *in vivo*. Pharmacological inhibition of MTHFD1 and/or MTHFD2 also impair tumour growth and improve survival in mouse models of leukaemia (8,31) colorectal (6) and breast cancer (7), which are discussed further below.

MTHFD2 may play also role in maintaining cancer stem cell populations. A common marker for stemness in cells is aldehyde dehydrogenase (ALDH) (32). MTHFD2 knockdown prevented sphere formation in ALDH high lung cancer cells (33), suggesting the cells cannot meet the 1C demands (e.g. nucleotide synthesis) to support cell proliferation and sphere formation. Although this does not exclude a role for MTHFD2 in initiation of growth in cancer stem cell populations. A role in maintaining cancer stem cells perhaps is logical given the normal function of MTHFD2 in embryonic development, where MTHFD2 helps to maintain pluripotency of stem cells (34).

Regulation of MTHFD2 expression in different cancer types

Overexpression of MTHFD2 across many cancer types is tied to its regulation by a number of different miRNAs and signalling pathways commonly associated with cancer. Despite many cancer types exhibiting MTHFD2 upregulation, so far distinct drivers of MTHFD2 overexpression have been identified in different cancer types. This is promising when developing new therapeutics, as many oncogenic transformations converge downstream at MTHFD2.

MicroRNAs play an important role in regulation of gene expression and can act as tumour suppressors in some instances (35). miR-33a-5p suppresses *MTHFD2* in colorectal cancer (36), miR-9 suppresses *MTHFD2* in breast cancer, (37) miR-940 suppresses *MTHFD2* in gliomas (38), and miR-92a/miR-504-3p suppresses *MTHFD2* and is in low abundance in AML patients to promote invasion, migration and inhibition of apoptosis via overexpressed MTHFD2 (39,40). Interestingly, miR-30a-3p not only seems to supress MTHFD2 in lung adenocarcinoma but miR-30a-3p levels are drastically different in lung adenocarcinoma cells and normal human bronchial epithelial cells (41). These differences in MTHFD2 miRNA regulation can possibly attribute the different MTHFD2 expression patterns in different cancer types. These miRNAs would function as MTHFD2 tumour suppressors, which when downregulated, result in overexpression of MTHFD2 in each cancer type.

A number of pathways with well-established roles in cancer are known to upregulate MTHFD2. Li *et al*. recently identified that MTHFD2 is transcriptionally repressed by the tumour suppressor p53. Inactivation of p53 led to upregulation of MTHFD2 and increased one-carbon folate metabolism and purine biosynthesis. This suggests that MTHFD2 may be an effective target in p53-null tumours. There are also links to oncogenic KRAS signalling via *c-myc* regulation of MTHFD2 expression (6,42). *c-myc* expression in colorectal cancer tissue is highly correlated with MTHFD2, and knockdown of both *KRAS* and *c-myc* in colorectal cancer cell lines resulted in suppression of MTHFD2 expression (6). In hepatocellular carcinoma cells, knockdown of nucleoside diphosphate kinase 7 (NME7), led to the

overexpression of MTHFD2 (43). This may be due to NME7 affecting the Wnt/β-catenin pathway as knockdown of NME7 resulted in decreased binding of β-catenin to the MTHFD2 promoter (43). These regulators of MTHFD2 expression are important in understanding how it behaves in different tumour types.

MTHFD proteins promotes an invasive phenotype and upregulation decreases cancer prognosis

MTHFD1 and MTHFD2 overexpression correlates with poor prognosis in multiple cancer indications. In lung adenocarcinomas, renal cell carcinoma, breast, colorectal and pancreatic cancer patients, elevated MTHFD2 had a strong correlation with decreased overall survival (OS) and disease-free progression (DFS) (44-48). This is consistent with mouse models showing MTHFD2 knockdown improved survival in mice with leukaemia (8,31). Moreover, poor prognosis is worsened when high *MTHFD2* expression is combined with overexpression of *SHMT2* and *ALDH1L2* (46). Expression of MTHFD2 is also highly cancer specific, with expression predominantly localized to the tumour tissue as opposed to the surrounding stromal tissue (5). MTHFD2 upregulation is, thus, characteristic to the tumour itself as opposed to alterations in the tumour microenvironment that promote tumorigenesis. The mechanisms to which MTHFD2 facilitates metastasis and poor prognosis is not fully understood, however, upregulation of mesenchymal characteristics and regulation of mitochondrial THF may be a part of this explanation.

The upregulation of mesenchymal characteristics promotes the invasiveness of cancer cells. Lin *et al*. found high MTHFD2 expression had a positive correlation with high vimentin expression in renal cell carcinoma patient samples (47). In breast cancer cells, knockdown of MTHFD2 and MTHFD1L resulted in weakened vimentin networks and decreased migration (49) and metastasis, which was driven by formate production (28). This is supported by *in vivo* studies showing knockdown of MTHFD2 in SW620 colorectal cancer cells results in fewer metastases in mouse xenograft models (6). There is evidence to suggest that MTHFD2 promotes EMT by the STAT3 pathway. Knocking down MTHFD2 in ovarian cancer cells not only decreased the expression of EMT markers, N-cadherin and vimentin, but overexpression of MTHFD2 resulted in increased expression of active phosphorylated STAT3 (p-STAT3) (50). This data supports a pro-metastatic role of MTHFD2 via EMT, but further studies are required to confirm the molecular mechanisms behind this.

MTHFD2 role in the DNA damage response pathway

MTHFD2 has a well-established role in mitochondrial 1C metabolism, however, recent studies have revealed distinct roles for nuclear MTHFD2. In 2015, MTHFD2 was shown to localise to the nucleus at areas of newly synthesized DNA and a screen of co-expressed genes also showed clustering of genes associated with DNA synthesis (51). This means that while mitochondrial MTHFD2 is implicated in synthesis of thymidine used in DNA synthesis, there may also be a direct nuclear role separate to the catalytic function in 1C metabolism.

MTHFD2 has a pivotal role in the DNA damage response (DDR), cell cycle arrest and protecting cancer cells from replication stress through supply of nucleotides. In non-small cell lung carcinoma (NSCLC),

upregulation of MTHFD2 increased cancer cell proliferation and MTHFD2 protein expression was directly proportional to Cyclin-A2 (CCNA2) which regulates G1/S and G2/M, the helicase Minichromosome Maintenance Complex Component 7 (MCM7) involved in G1/S transition and SKP2 expression (52). Inhibition of MTHFD2 using RNA interference induces DNA damage predominantly in S-phase of the cell cycle at replication forks in U2OS cells (8). DNA fiber assays revealed siMTHFD2 reduced fork speed, indicating impaired DNA synthesis and replication stress. The reduced viability induced by siMTHFD2 can be rescued by the addition of thymidine, or with an RNAi-resistant wildtype MTHFD2, but not catalytically-dead MTHFD2 (8). This neatly illustrates the importance of MTHFD2 in supply of thymidine for DNA synthesis required to sustain cancer cell growth.

In addition to preventing DNA damage, MTHFD2 may have further roles in maintaining genomic stability, by regulating DNA repair (Supplemental Figure 1). In mouse embryonic stem cells, nuclear MTHFD2 regulates homologous recombination (HR) to repair double strand breaks (DSBs) potentially through non-enzymatic functions (34). This occurs through MTHFD2 interaction with exonuclease 1 (EXO1) and cyclin dependant kinase 1 (CDK1), resulting in phosphorylation and thus stabilisation of exonuclease 1 (EXO1) to facilitate HR (34). Although this role has not yet been investigated in cancer, it may inform potential roles for MTHFD2 and HR in the future.

Recently, MTHFD2 was reported to function in non-homologous end-joining (NHEJ) (53). MTHFD2 was found to form a complex with Poly(ADP-ribose) polymerase 3 (PARP3), promoting formation of PARP3 tetramers and ADP ribosylation of PARP3 in response to DNA damage and promotes NHEJ. Similarly to HR, binding of MTHFD2 to PARP3 promotes NHEJ through a mechanism that is independent of the catalytic activity of MTHFD2 (53). Currently, the function of nuclear MTHFD2 in HR (34) and NHEJ (53) has only been shown in specific cell types (embryonic stem cells or p53 deficient cancer cells, respectively), and further studies are required to determine if these roles extend to other cell types.

MTHFD2 may also have implications in RNA synthesis and metabolism. Gene ontology of MTHFD2 revealed high co-expression with RNA processing elements, such as proteins in the heterogeneous nuclear ribonucleoprotein (hnRNP) family, and MTHFD2 physically interacted with many of these proteins (54). This revealed a novel function of MTHFD2 in RNA metabolism/translation, however the impact of these interactions in cancer are still unknown but could involve regulation of gene expression or a role in MTHFD2 control of proliferation and EMT.

Targeting MTHFD2 in treatment of cancer

The importance of one-carbon metabolism in cancer has led to the development of novel inhibitors. Dual inhibition of SHMT1 and SHMT2, by either shRNA or inhibitors such as SHIN-1 and AGF347, has shown promising anticancer activity *in vitro* and *in vivo* (55,56). Similar anti-tumour effects are observed with MTHFD1/2 inhibitors LY345899, DS18561882 and TH9619, but through different mechanisms (7,8). Studies to date have revealed that MTHFD2-high cancers can be targeted by direct inhibition of MTHFD2 and/or indirect inhibition of MTHFD1 (**Fig. 1B-C**).

In colorectal cancer, cell line xenografts (SW620, LoVo) and 2 patient-derived xenograft (PDX) models showed LY345899, an inhibitor of MTHFD1 and MTHFD2 resulted in decreased tumour volume as well as decreased metastasis to the intestine (6). Although LY345899 inhibits MTHFD2, it is reported to be more potent inhibitor against MTHFD1, IC50 of 663 nM vs 96 nM respectively (57). Simultaneously,

Kawai *et al*. developed another MTHFD1/2 inhibitor DS18561882. High dose (300 mg/kg) DS18561882 decreased tumour burden in the MDA-MB-231 breast cancer models *in vivo* with no change to mouse weight (7). Together, these studies confirm that pharmacological targeting of MTHFD2 is a promising anti-cancer strategy and provide strong incentive for translation into clinical trials.

The mechanism of DS18561882 is predominantly through blocking synthesis of purines. This means cells cannot produce the nucleotides required to replicate their DNA for cell division, and ultimately leads to growth arrest (**Fig. 1B**) (29). In colorectal cancer cells, supplementation with hypoxanthine almost entirely reverses DS18561882 toxicity (29). Whereas, purine deficiency is not the primary toxicity for TH9619 (which binds nuclear but not mitochondrial MTHFD2) (29). While this does not exclude a function for nuclear MTHFD2, it does suggest that nuclear MTHFD2 is not the major culprit contributing to tumorigenesis and as yet there is no evidence to support a therapeutic role as a single target, at least in cancer.

Treating MTHFD2-expressing cancers by inhibiting MTHFD1

Recently, our group identified a new approach to targeting MTHFD2-expressing tumours, by inhibiting MTHFD1 (29). Bonagas *et al*. performed a ligand-guided design to develop TH9619, which inhibits MTHFD2 and the DC domain of MTHFD1 in biochemical assays. TH9619 inhibited growth of leukemic HL-60 tumours through thymidylate depletion, and prolonged survival in mice on a low folate diet, performing better than standard-of-care cytarabine (8). Later it was discovered, that while TH9619 binds to nuclear MTHFD2 it does not bind mitochondrial MTHFD2 in SW620 colorectal cancer cells (29). Interestingly, it was demonstrated that TH9619 toxicity was due to inhibition of MTHFD1(DC) which resulted in trapping of the folate intermediate 10-formyl-THF. While TH9619 works by binding MTHFD1(DC), the folate trapping requires MTHFD2 to generate formate overflow. The accumulation of 10-formyl-THF leads to thymidylate depletion. With reduced thymidine, uracil is disincorporated into DNA during replication, causing DNA damage and apoptosis (**Fig. 1C**). Accumulation of 10-formyl-THF is exacerbated by physiological levels of hypoxanthine, which causes feedback inhibition on *de novo* purine synthesis, thus preventing consumption of 10-formyl-THF for purine synthesis. This revealed a novel way that MTHFD2 cancers can be exploited without directly targeting MTHFD2.

Considerations for MTHFD inhibitors

MTHFD2 lies within two membrane bound organelles, making the transport of drugs into the mitochondria and nucleus an important factor to consider. For classical antifolates (which are structurally similar to folate), they must be polyglutamylated by folypolyglutamate synthetase to be retained within the cell and its downregulation leads to anti-folate resistance (58,59). Interestingly, the folate-like inhibitor TH9619 binds nuclear but not mitochondrial MTHFD2, presumably because it is not being transported into the mitochondria (29). In comparison, DS18561882, which is structurally distinct from folate, does inhibit mitochondrial MTHFD2 (29). Thus, some challenges in targeting mitochondrial MTHFD2 may be avoided through design of therapeutics that are structurally distinct from folate intermediates.

One of the greatest challenges when designing MTHFD2-specific inhibitors is the structural similarity to MTHFD2L and the dehydrogenase/cyclohyrolase (DC) domain of MTHFD1 (60). Despite the compartmentalisation of MTHFD1 and MTHFD2, the cytosolic MTHFD1 is easier to access and thus a MTHFD2 inhibitor will likely also inhibit MTHFD1.

The putative switch to SHMT1 mediated synthesis of nucleotides in absence of MTHFD2 may be another limitation to the efficacy of MTHFD2 inhibitors (16). Many reports validate MTHFD2 as a target (**Table 1**), contrasting with The Cancer Dependency Map Project, where genome-wide CRISPR-Cas9 screening suggests *MTHFD2* is a non-essential gene and not required for cancer cell growth (12). A simple explanation for *MTHFD2*$^{-/-}$ cells being able to survive is that they switch to SHMT1 for synthesis of thymidine and purines (16). Current MTHFD inhibitors are able to circumvent this switch, through dual inhibition of MTHFD2 and MTHFD1 (DC), which would block purine synthesis by both the mitochondrial and cytosolic routes.

MTHFD2 cancer-specificity and potential inhibitor side effects

Cancer cells have a higher demand for nucleotides to support DNA replication and cell division, which is supported by increased MTHFD2 expression. The main functions in normal biology, are in embryonic development and T cell activation, as discussed earlier. The role in T cell activation, suggests MTHFD2 inhibitors may also have anti-inflammatory properties. In the context of cancer, investigation is required to identify the impact of MTHFD2 inhibitors on immune response and whether this will be a problem in patients who are often immunosuppressed due to other treatments or the cancer itself.

What remains to be elucidated is whether the cancer-specific expression of MTHFD2, will lead to development of new, effective treatments with fewer side effects in patients. Anti-folates (e.g. methotrexate, pemetrexed) and 5-FU are effective chemotherapeutics that have been around for decades, but they are not cancer-specific and hit multiple targets, resulting in side-effects commonly associated with chemotherapeutics including gastrointestinal symptoms, fatigue and hair loss. The targets of anti-folates, DHFR, TYMS, glycinamide ribonucleotide formyl transferase (GARFT), 5-amino-4-imidazolecarboxamide ribonucleotide transformylase (AICART) and amidophosphoribosyltransferase (ATase), are also expressed by normal cells, and thus methotrexate (and 5-FU which inhibits TYMS) also cause DNA damage in healthy cells, which is not observed with TH9619 (8). Whether the targeted action of TH9619 translates into fewer adverse effects in the clinic, will likely be discovered in the coming years as clinical trials commence with these targeted inhibitors.

Mice are not ideal models for one-carbon therapeutics

An important consideration highlighted by Bonagas *et al* and Green *et al*. is that mice are not an ideal model of humans when it comes to one-carbon metabolite levels (8). Thymidine and folate levels are both ~100 fold higher in mice than humans, and hypoxanthine levels are 100-1000 fold higher in mice than humans (22,23). This has important implications for preclinical testing of new drugs targeting MTHFD2-expressing cancers. DS18561882, predominantly works by inhibiting purine synthesis and inhibiting mitochondrial MTHFD2 (29), and inhibits tumour growth in breast cancer mouse models (61). Given hypoxanthine rescues DS18561882 toxicity, and hypoxanthine levels are higher in humans than mice, it is important to consider whether responses to direct MTHFD2 inhibition in mice are

translatable to patients. In comparison, TH9619 works by causing thymidylate depletion and was only effective in mice with HL-60 tumours when they were fed a low folate diet to reduce folate levels (8). Yet, in human phycological medium (human plasma like-medium (22) or Plasmax (62), viability was reduced in colorectal cancer cells at low nM concentrations of TH9619, and sphere growth reduced in 3D culture (29). The impact that these metabolite differences between mice and humans will have on translation of drugs targeting MTHFD2 (or indeed MTHFD1 to target MTHFD2-expressing cancers) into patients remains unclear but may pose as an important consideration for the development of one-carbon metabolism inhibitors in the future.

Combination therapies & biomarkers

Given the complex treatment landscape across cancer types, it is likely that future clinical use of MTHFD1/2 inhibitors will include combination with existing and novel therapies and these may differ by cancer type. MTHFD1/2 inhibition *in vitro* and *in vivo* demonstrates that it can be an effective as a single agent. However, a number of targets and compounds have been shown to improve MTHFD1/2 inhibitors.

One potential target is PAICS, a member of the purinosome involved in *de novo* purine synthesis. In MYCN neuroblastoma cell lines, dual knockdown of MTHFD2 and PAICS by shRNA significantly decreased proliferation and colony formation compared to single knockdown (63). One carbon metabolismproduces 10-CHO-THF used in purine synthesis, and PAICS produces phosphoribosylaminoimidazolesuccinocarboxamide (SAICAR) in *de novo* purine synthesis. This means that dual targeting of PAICS and MTHFD2 would prevent production of both precursors required to synthesise purines.

Ducker *et al* found that in *MTHFD2*$^{-/-}$ cells 1C flux reversal occurred, where thymidine and purines were produced via cytosolic SHMT1, rather than from formate produced by mitochondrial MTHFD2 (16). SHMT1 may thus provide resistance to MTHFD2 inhibitors. Indeed, knockdown of MTHFD2 and SHMT1 via shRNA resulted in complete inhibition of cancer cell proliferation and a double MTHFD2/SHMT1 deletion HCT-116 cell line resulted in no tumour growth *in vivo* (16). Thus, combining a MTHFD2 with a SHMT1 inhibitor may improve anti-tumour activity and/or overcome resistance in some tumours.

TH9619 has been shown to synergise with dUTPase inhibitors (8). During thymidine synthesis TYMS produces dTMP and dihydrofolate from dUMP and 5,10-CH_2-THF. The enzyme dUTPase hydrolyses dUTP to dUMP, to prevent uracil incorporation into DNA. Using TH9619 and compounds earlier in the series, remarkable synergy was exhibited with dUTPase inhibitors, which could be rescued with the addition of thymidine.

DS18561882 enhanced the anti-tumour properties of a checkpoint kinase 1 (Chk1) inhibitor. The Chk1 inhibitor overcame the growth arrest caused by DS18561882, leading to accumulation of DNA damage and apoptosis which prevented tumour growth (64). Achreja *et al*., found that ovarian cells with a loss of *UQCR11,* required for regulating complex III of the electron transport chain (ETC), upregulates

MTHFD2 to compensate for alterations of the NAD+/NADH ratio alterations caused by ETC impairments (65). DS18561882 treatment of *UQCR11*-null ovarian cells improved sensitivity by six-20 fold compared to *UQCR11*-inact cells.

Identifying biomarkers for MTHFD inhibitor use is also important for clinical translation. MTHFD2 has a wide tumour type expression, and establishing proper genetic profiles is essential in determining which populations would be the most sensitive to MTHFD2 inhibition. Oncogenic KRAS has been identified as a driver of MTHFD2 expression in cancer (6,66). As such, KRAS activity may predict MTHFD1/2 inhibitor sensitivity.

Conclusion and Future Directions

MTHFD2 has well established roles in cancer development and prognosis. MTHFD1(DC) and MTHFD2 inhibitors are now in development and are exhibiting promising anti-cancer activities in pre-clinical models. The potential non-catalytic roles for MTHFD2 raise the question of whether these functions will be impacted by current MTHFD2 inhibitors, or perhaps whether protein interactions can be disrupted independently of the catalytic function by designing new compounds. Moreover, designing a highly selective MTHFD2 inhibitor will be challenging given the structural similarities between MTHFD2, MTHFD1(DC) and even MTHFD2L. As such, it will be important to consider whether dual targeting of MTHFD2 and MTHFD1 will be beneficial or deleterious in the treatment of cancer patients.

Table 1. Summary of MTHFD2 *in vivo* cancer experiments

Study	Mouse model	Summary
Moran et al, 2014 (42)	KRAS-mut NSCLC and KRAS-WT model, nude mice	• MTHFD2 expression directly proportional to KRAS expression • Tumours with higher MTHFD2 expression had increased sensitivity to TYMS inhibitor, pemetrexed
Pikman et al., 2015 (31)	1) Human orthotopic xenograft AML 2) MLL-AF9 leukemia model, C57BL/6 mice	• *MTHFD2* knockdown improves survival and decreases tumour burden in mice
Gu et al., 2017 (39)	HL-60 AML model	• Upregulation of miR-92a inhibits *MTHFD2* and decreased tumour size compared to controls
Xu et al., 2019 (38)	U87 Glioma xenograft model, Male BALB/c nude mice	• Overexpression of miR-940 supresses *MTHFD2* and results in decreased tumour volume
Ju et al., 2019 (6)	Colorectal PDX, Female BABL/c nude mice	• *MTHFD2* inhibition by LY345899 decreases tumour volume and metastasis
Yan et al., 2019 (36)	HCT116 colorectal, Male BALB/c nude mice	• Mice with miR-33a-5p expression decreased tumour size and slowed tumour growth rate via suppression of *MTHFD2*

Kawai et al., 2019 (7)	MDA-MB-231 Breast cancer xenograft model, BALB/cAJcl-nu/ nu mice	• DS18561882 significantly reduced tumour growth but only at 300 mg/kg and had no alteration to mouse body weight
Yu et al., 2020 (52)	H1299 NSCLC xenograft model, female BALB/c nude mice	• Knockdown of *MTHFD2* resulted in decreased tumour volume and weight
Lee et al., 2021 (20)	Triple negative breast cancer PDX model, NSG mice	• DS18561882 plus Chk1 inhibitor, LY2606368, decreased tumour volume
Bonagas et al., 2022 (8)	HL-60 Leukemia xenograft, NOD.CgPrkdcscidIl2rgtm1Sug/JicTac (NOG)	• TH9619 treatment with a low-folate diet increased survival by 2 weeks
Acherja et al., 2022 [(65)]	*UQCR11*-null or *UQCR11*-intact OVCAR8 and SKOV3 ovarian xenograft model	• Dox-induced knockdown of MTHFD2 improved inhibition of tumour growth and increased apoptotic cell population in tumour samples

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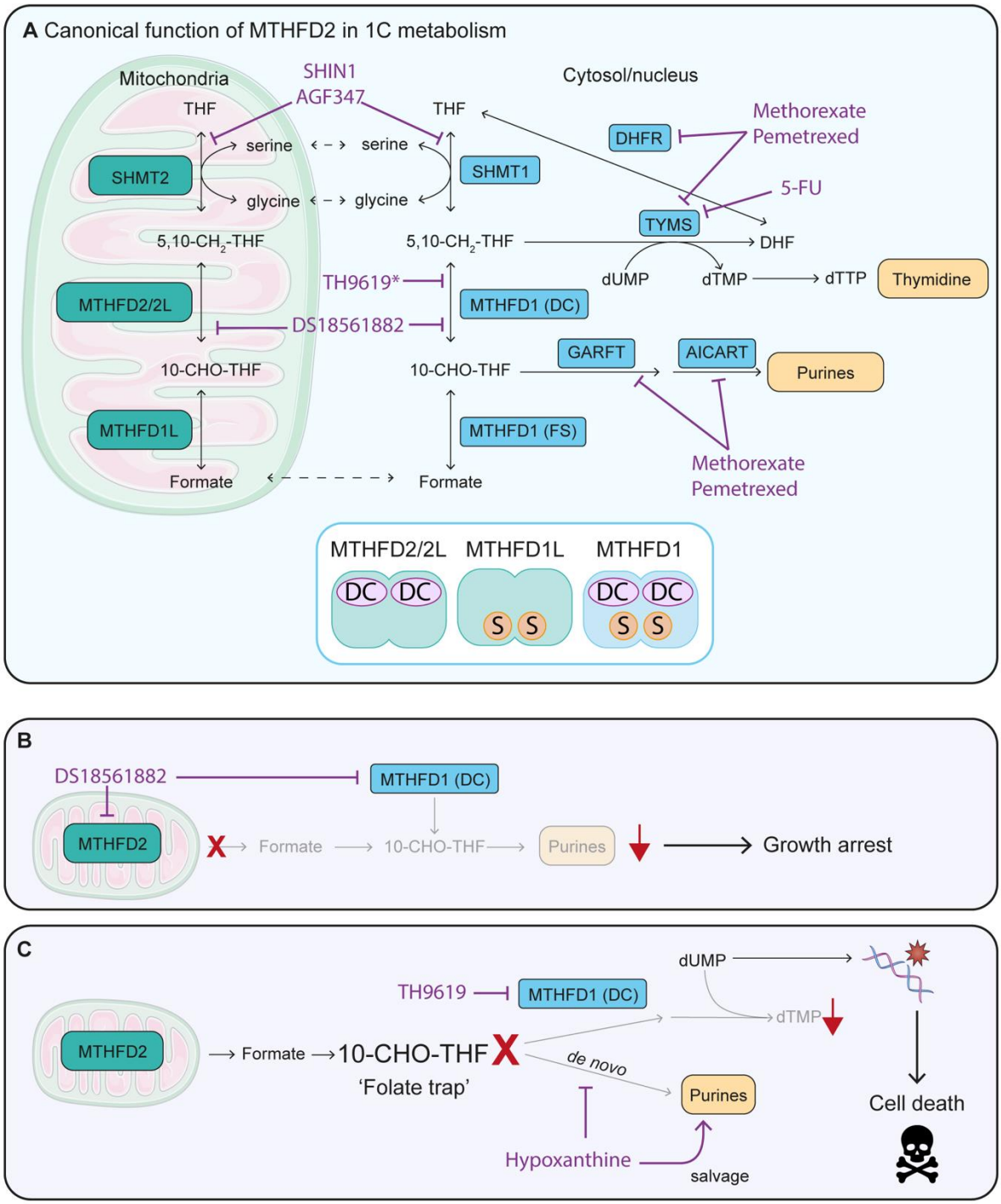
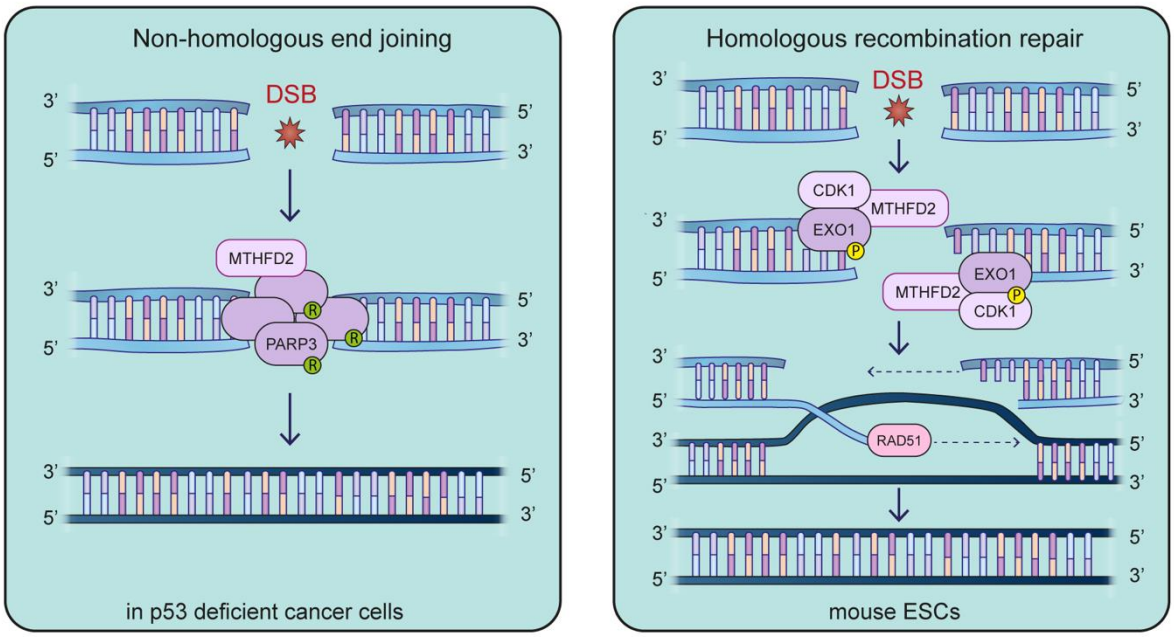


Figure 1.

Figure 1. (**A**) Schematic of one-carbon metabolism and pathway inhibitors. One-carbon metabolism Is compartmentalised between the mitochondria, cytosol and nucleus, the pathways are bi-directional but in cancer formate is predominantly produced via the mitochondria. Mitochondrial enzymes serine hydroxymethyltransferase (SHMT2), methylenetetrahydrofolate dehydrogenase/cyclohydrolase (MTHFD)2/2L and MTHFD1L interconvert folates and formate, whereas in the cytosol/ nucleus this is performed by SHMT1 and the tri-functional enzyme MTHFD1 which contains a dehydrogenase/cyclohydrolase (DC) domain and a formyl-THF synthetase domain (S). This cycle produces 5, 10-CH_2-THF which is used to create thymidine and 10-CHO-THF used in purines synthesis. While there are some

differences in structure and amino acid sequences, the DC domains of MTHFD2/2L/1 and the S domain of MTHFD1/1L are highly similar. Compounds targeting one-carbon enzymes, 5-fluorouracil (5-FU) and anti-folates (methotrexate, pemetrexed) are indicated in purple. *TH9619 can also inhibit MTHFD2 in biochemical assays but does not inhibit mitochondrial MTHFD2, presumably because it does not enter the mitochondria. (**B**) Mechanism of action of MTHFD2 inhibitor DS18561882. Inhibition of MTHFD2 and MTHFD1 depletes formate and prevents purine synthesis. Depletion of purines means cells lack the nucleotides required for replication of DNA and proliferation, thus causing growth arrest. (**C**) Inhibition of MTHFD1 (DC) by TH9619 results in accumulation of 10-CHO-THF (Folate trap). In turn, the decreased thymidine production leads to misincorporation of uracil into DNA, causing genomic instability and thereby resulting in cell death.



Supplemental Figure 1. An overview of the roles of MTHFD2 in DNA damage repair. (**A**) In the event of a double stranded break (DSB), MTHFD2 forms a complex with PARP3 to facilitate homologous end joining and (**B**) with EXO1 and CDK1 in mouse embryonic stem cells (ESCs) to facilitate homologous recombination.