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NCI, DCPC
Chemoprevention Branch and Agent Development Committee

CLINICAL DEVELOPMENT PLAN: FOLIC ACID

DRUG IDENTIFICATION

CAS Registry No.: 59-30-3

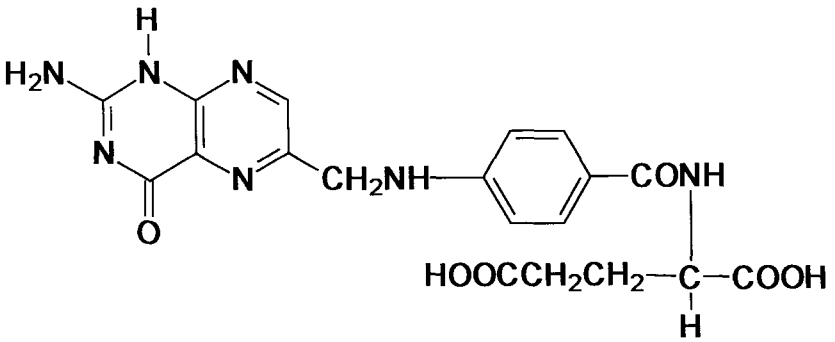
CAS Name (9CI): N-[4-[[(2-Amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]benzoyl]-L-glutamic Acid

Synonyms:
Cytofol
Foldine
Liver *Lactobacillus casei* Factor
Pteroylglutamic Acid (PGA)
Vitamin B Complex
Vitamin M

Related Compounds:
Methyltetrahydrofolate
Folinic Acid (Citrovorum Factor)
10-Formyltetrahydrofolate
5,10-Methenyltetrahydrofolate
5,10-Methylenetetrahydrofolate
Formiminotetrahydrofolate
Hydroxymethyltetrahydrofolate

Molecular Wt: 441.4

Structure:



EXECUTIVE SUMMARY

Folic acid is a water-soluble B complex vitamin. It consists of a pteridine moiety linked to *para*-aminobenzoic acid and L-glutamate. Folic acid is the commercially available form of folate used in vitamin supplements. Folate is a generic term describing the many different forms of the vitamin possessing biological activity. Folate cannot be synthesized by mammals and therefore must be supplied in the diet by foods such as liver, leafy green vegetables, legumes and some fruits. Dietary sources of folate are generally polyglutamates, although only the mono-

glutamate can be absorbed directly from the intestine [1]. The intestinal mucosa of animals contain enzymes known as conjugases which remove all but the last glutamate residue. The monoglutamate folates are than taken up by the brush border, reduced to tetrahydrofolate, and methylated to methyltetrahydrofolate, the metabolically active form of folate [1]. This is the predominant form of folate in plasma, the majority of which is bound to protein.

The current US recommended dietary allowances of 200 μg/day (*ca.* 6.5 nmol/kg-bw/day) for adult males, 180 μg/day (*ca.* 6.4 nmol/kg-bw/day) for adult females, and 400 μg/day (*ca.* 14.3 nmol/kg-bw/day) during pregnancy are being re-evaluated as possibly too low [1,2]. Low plasma folate is associated with elevated levels of homocysteine, a known risk factor for coronary artery disease [3]. It has also been well established that folic acid supplementation before and during pregnancy can prevent 75% of fetal neural tube defects [4]. In 1996, the FDA mandated fortification of flour and wheat grains with 140 μg folic acid per 100 g of grain to help protect against these birth defects.

Folates are coenzymes involved in the transfer and utilization of one-carbon units in a variety of essential reactions, including amino acid metabolism, biosynthesis of DNA and RNA, and biosynthesis of *S*-adenosylmethionine (SAM). SAM is the universal methyl donor and participates in the methylation of proteins, DNA, phospholipids and small molecules. Methylation of DNA occurs on the fifth carbon of the pyrimidine ring of cytosine residues in CpG dinucleotide sequences. It occurs following DNA replication by a maintenance DNA methyltransferase, thus preserving the methylation pattern in the newly synthesized DNA strand [5,6]. Since DNA methylation plays an important role in inhibiting gene transcription, failure to maintain the methylation pattern of nascent DNA may facilitate the aberrant expression of oncogenes, thereby contributing to carcinogenic progression. Indeed, hypomethylation of DNA is one of the most consistent alterations of the genome observed in carcinogenesis [7,8]. In particular, DNA hypomethylation is considered to be an early event in colon and cervical cancer [9,10]. It has been shown to increase during the histopathologic progression from normal to invasive disease [8]. In a recently published study, Cravo *et al.* demonstrated that DNA from the rectal mucosa of patients with colorectal neoplasms is globally hypomethylated compared with controls, and that hypomethylation is significantly reduced with folic acid treatment [11].

In contrast, regional hypermethylation of DNA has been reported to play a role in carcinogenesis, presumably by inhibiting transcription of certain tumor suppressor genes [12]. Distinct patterns of DNA hypermethylation have been shown to occur in human lung, colon, breast and prostate carcinomas [13–16]. Indeed, abnormal hypermethylation of the GST-π promoter is the most common genetic defect identified in prostate cancer to date and may contribute to the pathogenesis of the disease by inhibiting the activity of this carcinogen detoxification enzyme [16]. Thus, the role of DNA methylation in carcinogenesis is clearly complex and has yet to be fully delineated.

In preclinical studies, rats maintained on methyl-deficient diets (deficient in folic acid, choline and methionine) develop fatty livers and liver tumors. Prolonged intake of a methyl-deficient diet results in depletion of SAM pools which leads to DNA hypomethylation with resultant altered gene expression and an increase in DNA strand breaks [17,18]. In addition, under conditions of low levels of SAM, DNA methyltransferase can catalyze the deamination of cytosine to form uracil resulting in transition from a C-G base pair to a T-A pair [19,20]. It is known that more than 25% of the point mutations in the p53 tumor suppressor gene occur at CpG sites and that five of six mutational hotspots in this gene are CpG sites where the cytosines are normally methylated [21–25]. Animal studies have shown that a diet severely deficient only in folic acid can also cause hypomethylation of DNA in rat livers [26]; although, another study reported that moderate folate deficiency does not cause a significant change in global methylation of rat liver or colon DNA [27]. One study reported that a diet severely deficient only in folic acid enhanced the development of carcinogen-induced colon tumors in rats [28]. Folic acid supplementation was shown to inhibit carcinogen-induced lung tumors in mice and respiratory tract metaplasia in rats. In contrast, several NCI-sponsored studies reported no effect of folic acid treatment on the development of carcinogen-induced colon tumors or aberrant crypt foci in rats or against carcinogen-induced mammary tumors in rats. Thus, results from animal studies are conflicting, suggesting that effects of folic acid on carcinogenesis are complex and may depend on several factors, including animal model, tumor type, timing, dose and duration of carcinogen

treatment, route of administration of folic acid, and the folate status of the animal.

Epidemiological studies have established an association between low dietary and/or low serum or red blood cell (RBC) folate levels and the incidences of colon and cervical cancer [29]. There is strong epidemiological evidence suggesting that a diet low in folate is associated with increased incidence of colorectal adenoma and cancer [30–34]. This is supported by the finding that patients with ulcerative colitis receiving folate supplementation had a significantly lower incidence of colorectal dysplasia and neoplasia compared with individuals not receiving supplements [35]. In addition, several recent studies reported that the RBC folate level in patients with colorectal adenomas and cancer was significantly lower than controls and a concentration below 160 ng/ml is associated with increased risk of developing colon cancer [36–38]. However, these studies could not determine whether low RBC folate levels are a cause or a consequence of the disease.

Two prospective epidemiological studies reported an inverse association between RBC folate levels and cervical cancer [39,40]. One study reported that a RBC folate concentration below 140 ng/ml is associated with increased risk for CIN [39]. Use of oral contraceptives is known to interfere with folate absorption, and the reduced RBC folate levels are associated with an increased risk for CIN [39]. In contrast, another study reported no significant association between RBC folate levels and CIN, although low RBC folate levels were associated with an increase in human papilloma virus infection, a known risk factor for CIN [41]. When folate status was determined either by diet or serum folate levels, five of fifteen studies showed a significant correlation between low folate levels and increased incidence of cervical cancer or CIN [29–48].

Several studies have also reported an association between low RBC and/or serum folate levels and the incidence of esophageal cancer [29,49]. In contrast, a recent study reported no association between breast cancer risk and intake of folic acid supplements [50]. In two epidemiological studies, no significant association was found between folate intake and lung cancer [51,52]. Although the results of these studies are not completely consistent, it must be remembered that they differ greatly in their methods and timing of folate status assessments; specifically, none have assessed folate status in the target organ of interest during the neoplastic process. In addition, these studies differ in their disease endpoints (*e.g.*, cancer versus preinvasive neoplasia). Taken together, the results of these studies suggest that low folate is associated with an increased risk for neoplastic disease, even in early stages of the pathophysiologic process. Because of the weight of the epidemiological evidence, preliminary clinical intervention data and the lack of toxicity, the NCI, Chemoprevention Branch undertook development of folic acid as a cancer chemopreventive agent. Based on the available data, the primary targets were the gastrointestinal tract and cervix.

The NCI, Chemoprevention Branch funded one Phase II and one Phase III placebo-controlled clinical trials with folic acid for the prevention of colorectal cancer. Both of these trials are in progress. The Phase II trial (Dr. Joel B. Mason, Tufts University) is designed to evaluate the efficacy of folic acid in patients with colorectal adenomas. Patients are administered 5 mg folic acid qd (0.2 μmol/kg-bw/day) orally for 24 months, then followed for an additional 12 months. The incidence and regression of polyps will be monitored and effects on DNA methylation will be assessed as well. The Phase III trial (Dr. John A. Baron, Dartmouth College) is designed to investigate the efficacy of folic acid on the recurrence of neoplastic colon polyps in patients with a history of colorectal adenomas. Patients are administered 1 mg folic acid qd (0.03 μmol/kg-bw/day) orally with and without two dose levels of aspirin for three years using a 2x3 factorial design.

The NCI, Chemoprevention Branch has funded one placebo-controlled Phase III clinical trial (Dr. Joseph Chu, University of Washington, Seattle) to investigate the effect of folic acid treatment on the regression of CIN lesions [53]. In this completed study, patients with mild or moderate CIN were administered 5 mg folic acid/day (0.2 μmol/kg-bw/day) or placebo orally for six months. There was no significant difference in the improvement of CIN between the two groups [53]. In an earlier study, Whitehead *et al.* reported that folic acid supplementation reversed megaloblastic abnormalities of cervical epithelia related to oral contraceptive use [54]. Additionally, in a small uncontrolled clinical trial with patients possessing mild or moderate CIN, those receiving 10 mg folic acid/day (0.4 μmol/kg-bw/day) orally for three months showed a significant improvement in biopsy scores [55]. A larger subsequent trial

conducted by the same group over a six-month period did not reveal any significant improvement following folic acid treatment [56]. Since these studies were performed on patients with established lesions it is unclear whether folic acid treatment might be efficacious if given prior to the development of cervical dysplasia. In contrast, oral administration of 10 to 20 mg folic acid/day (0.3 to 0.6 μmol/kg-bw/day) in combination with vitamin B_{12} for up to one year was shown to be effective in reducing bronchial squamous metaplasia in smokers [57,58]. Further clinical studies await the outcome of the ongoing Phase II and Phase III trials.

The NCI, Chemoprevention Branch has not funded any toxicity studies on folic acid, but it is regarded as nontoxic in humans and animals. However, there is a concern that in correcting the anemia associated with vitamin B_{12} deficiency, folic acid may mask the underlying deficiency and increase the risk of permanent neurological damage. This uncertain risk can be addressed by testing for vitamin B_{12} deficiency and providing a supplement if necessary. Folic acid also inhibits the absorption of phenytoin, a drug used for the treatment of epilepsy. These patients should be excluded from any folic acid trials.

There should be no problems with the supply of folic acid since it is commercially available in the US in oral tablet form. For future blinded studies, the availability of a suitable placebo will need to be determined.

PRECLINICAL EFFICACY STUDIES

The effects of folic acid on carcinogenesis in animals are conflicting and controversial. According to some studies, methyl-deficient diets and diets deficient only in folic acid result in hypomethylated DNA and increased expression of certain oncogenes in rat liver [17,18, 26]. In one study, rats maintained on a folic acid-deficient diet showed enhanced development of DMH-induced colonic neoplasia [28]; in another study, rats maintained on a diet moderately deficient in folic acid did not show a significant reduction in global DNA methylation in liver or colonic mucosa or in c-*myc*-specific colonic mucosal DNA methylation [27]. Results from animal studies investigating the effects of supranutritional folic acid treatment on carcinogen-induced tumorigenesis are also conflicting. In NCI, Chemoprevention Branch-sponsored studies, folic acid was not effective against carcinogen-induced rat and mouse colon, rat mammary gland, mouse urinary bladder and hamster lung tumors. Folic acid supplementation for 30 days did not prevent MNU-induced mammary tumors in rats; on the contrary, it resulted in early appearance of tumors [59]. In published studies, folic acid was shown to be effective in inhibiting carcinogen-induced bronchial squamous metaplasia in rats and lung tumors in mice [60,61]. In another study, supplementing the diet with 40 mg folic acid/kg (0.5 μmol/kg-bw/day) for up to 20 weeks had no effect on DEN-induced hepatocarcinogenesis in male Wistar rats [62].

A recent NCI, Chemoprevention Branch-sponsored study examined the effects of folic acid on AOM-induced colon carcinogenesis in rats. Male F344 rats were fed either control diets or diets containing 2.0 g folic acid/kg (227 μmol/kg-bw/day) beginning two weeks before AOM treatment. Folic acid had no significant effect on colon tumor incidence; in fact, it increased tumor size and multiplicity when compared with animals on the control diet [63]. In another NCI, Chemoprevention Branch-sponsored study, female CF-1 mice were treated with MAM acetate for six weeks. Animals were treated with 25 or 50 g folic acid/kg diet (7.0 or 15 mmol/kg-bw/day) for 40 weeks. Folic acid was reported to have no effect on colon tumor formation. In other NCI, Chemoprevention Branch-sponsored studies, 2.5 and 5.0 g folic acid/kg diet (*ca.* 283 and 566 μmol/kg-bw/day) had no effect on MNU-induced mammary gland tumors in female Sprague-Dawley rats and 0.5 and 2.0 g folic acid/kg diet (*ca.* 146 and 582 μmol/kg-bw/day) had no effect on OH-BBN-induced urinary bladder cancer in male BDF mice.

Animal studies have also investigated the modulation of intermediate biomarkers of cancer by folic acid. In an NCI-sponsored study, the effect of folic acid on AOM-induced aberrant crypt foci in rats was examined. The foci are aggregates of one or more dysplastic crypts and are considered premalignant lesions. Male F344 rats were administered either 2.5 or 5.0 g folic acid/kg diet (*ca.* 283 or 566 μmol/kg-bw/day, respectively) starting one week prior to AOM treatment and continuing for four weeks. It was determined that folic acid had no significant effect on AOM-induced aberrant crypt foci in the rat colon when compared with control animals [64]. However, in an *in vitro* study, 500 ng/ml (1.1 μM) folic acid inhibited MAM-induced ODC and tyrosine kinase activity, markers of cellular proliferation, in colorec-

tal mucosal explants of rats [65].

Folic acid was shown to be effective in inhibiting carcinogen-induced bronchial squamous metaplasia in rats and lung tumors in mice [60,61]. Sprague-Dawley rats maintained on a folate-containing diet were administered two doses of 5 mg methylcholanthrene (MCA) intratracheally separated by one day. Fifteen mg folic acid (113 μmol/kg-bw) were administered intramuscularly twice a week starting nine weeks after MCA treatment and continuing for 28 weeks. Serum folate levels decreased from 60 ng/ml prior to MCA treatment to 20 ng/ml at the end of the study for the carcinogen control group, while folate levels for the folic acid-treated group remained unchanged. Folic acid treatment reduced both the degree and incidence of MCA-induced metaplasia in the rat airway epithelium [60]. In another study, Swiss mice were treated with isoniazid (1.1 mg/mouse) six days a week by gavage with or without 1.1 mg folic acid (71 μmol/kg-bw/day by gavage) for up to 24 months. The authors reported a complete inhibition of lung tumor incidence in the folic acid-treated mice [61]. In a similar study by the same group, folic acid was reported to be marginally effective against hydrazine sulphate-induced lung tumors in Swiss mice [66]. In an NCI, Chemoprevention Branch-funded study, 1 or 2 g folic acid/kg diet (*ca.* 272 or 544 μmol/kg-bw/day) had no effect on DEN-induced lung tumors in male Syrian hamsters.

Thus, animal studies suggest that the efficacy of folic acid in prevention of carcinogenesis depends on several factors, including the animal and tumor model; the type, timing and dose of carcinogen; and the dose, route and timing of folic acid administration. It is important to note that folic acid was not found to be effective against carcinogen-induced tumors in animal studies when administered in the diet. Another critical confounding factor in studies of the preventive or therapeutic efficacy of folic acid supplementation may be the baseline folate status of the animal. Relative to humans, rodents have folate-replete diets; the rat consumes approximately 0.3 mg folic acid/kg-bw/day (0.7 μmol/kg-bw/day) in the normal rodent diet, a level 100-fold higher than the recommended dietary allowance for humans. The results of the animal studies suggest that low folate levels, whether a result of dietary folate deprivation or chemically induced reductions in folate status via MCA or isoniazid administration, create a permissive environment for carcinogenesis which might be corrected by folic acid supplementation. These same supplements in the presence of normal dietary folate intakes may not prevent carcinogenesis, and may even promote neoplastic progression [28,60,61].

PRECLINICAL SAFETY STUDIES

Safety: There are no published preclinical toxicity studies with folic acid. It is considered nontoxic at high doses in both humans and animals. The MTD relating to oral administration has not been established. In some laboratory animals, very high doses of folic acid given parenterally may precipitate in the kidneys causing kidney damage and hypertrophy [2]. No teratology or carcinogenic studies for folic acid were identified, but folic acid was shown not to be mutagenic towards *Salmonella typhimurium* strain TA100 [67,68].

ADME: The NCI, Chemoprevention Branch has not sponsored any preclinical pharmacokinetic studies on folic acid. In a recently published study, the enterohepatic circulation kinetics of folic acid was investigated in rats. Under conditions of bile diversion, the authors reported a significant decrease in plasma folate concentrations over 5 hours. These results suggest that enterohepatic circulation is important for maintaining plasma folate levels in the rat [69].

CLINICAL SAFETY: PHASE I STUDIES

The NCI, Chemoprevention Branch has not sponsored any Phase I trials with folic acid. It is considered to be nontoxic in humans, with a long history of human consumption. The recommended dietary allowance, based on prevention of known deficiency syndromes in the US population, is 200 μg/day (*ca.* 6.5 nmol/kg-bw/day) for adult males, and 180 μg/day (*ca.* 6.4 nmol/kg-bw/day) for adult females, increasing to 400 μg/day (*ca.* 14.3 nmol/kg-bw/day) during pregnancy [1,2]. There have been several pharmacokinetics studies published and results from these studies are summarized below.

Drug Effect Measurement: Traditionally, folate concentrations in biological fluids have been measured using microbiological growth assays, though the presence of antibiotics and other drugs in the sample can influence the results. More precise detection methods are HPLC, radioisotope dilution competition binding assays and GC-MS, although these analytical methods depend on a reliable internal standard for accurate determination of folate concentration

[70–72]. The RBC folate concentration is considered a better indicator of global tissue folate stores than serum folate levels, since the latter fluctuate greatly and can take up to several weeks to stabilize under controlled conditions of intake.

During methylation reactions, SAM is converted to *S*-adenosylhomocysteine (SAH). Folate status has been shown to influence the SAM/SAH ratio in certain tissues; therefore, SAM/SAH ratio may be a potential drug effect measurement for folic acid [73]. In addition, low levels of serum and tissue folate are often associated with increased levels of serum homocysteine. Homocysteine levels decrease in response to folic acid treatment; therefore, serum homocysteine levels are another potential drug effect measurement [3]. Since folic acid treatment has been shown to increase DNA methylation in the rectal mucosa of patients with colorectal adenomas [11], monitoring global DNA methylation may be an additional drug effect measurement for folic acid.

Safety: Folic acid is essentially nontoxic in humans. In clinical studies, no adverse effects were reported in women taking 10 mg folic acid/day (*ca.* 0.4 μmol/kg-bw/day) continuously for four months, a dose equivalent to >50 times the RDA [56]. Daily oral doses of 15 mg folic acid (*ca.* 0.54 μmol/kg-bw/day for females and 0.48 μmol/kg-bw/day for males) also showed no adverse effects [74]. Only a few cases of allergic reactions to folic acid have been reported [74]. There is some concern that folic acid may correct the anemia associated with vitamin B_{12} deficiency, and thereby delay the diagnosis and treatment of this deficiency, increasing the risk of permanent neurological damage [4,75]. This can be addressed by testing for this deficiency and providing a supplement if necessary. Inclusion of up to 1 mg vitamin B_{12} in vitamin supplements containing 400 μg folic acid has been recommended to protect vitamin B_{12}-deficient individuals [3]. Folic acid inhibits the uptake of the anticonvulsant drug phenytoin at the gut cell membrane and possibly at the brain cell membrane. Very high doses of folic acid (>20 mg/day) may cause convulsions in persons whose epilepsy is in continuous control by phenytoin [2,76]. There is also some evidence suggesting that high doses of folic acid for long periods of time may interfere with zinc absorption [1].

ADME: The NCI, Chemoprevention Branch has not sponsored any Phase I pharmacokinetics studies with folic acid. According to published information, dietary folates (often polyglutamates) are absorbed by the proximal intestinal mucosa after being converted to the monoglutamate form by conjugase enzymes in the bowel lumen and intestinal mucosa [77]. Following absorption, the majority of folate in the circulation is loosely bound to protein. Tetrahydrofolic acid and its derivatives are found in all body tissues, but the liver contains the majority of the total body folate stores [76]. In a recent published pharmacokinetics study, healthy adult volunteers were given 5 mg folic acid (*ca.* 0.2 μmol/kg-bw) by oral or iv administration in a two-way crossover trial. Folic acid was measured in the serum and urine over a twelve-hour period following treatment. Postdose levels of folic acid were corrected with the individual's predose levels. After oral administration, the C_{max} was 243±33 ng/ml at a t_{max} of 2.24± 0.85 hours. The mean AUC was 1,160±177 ng•hr/ml. Following iv administration, serum levels ranged from 559 to 1,490 ng/ml at a t_{max} of six minutes. The AUC was 1,550±249 ng•hr/ml. Following oral administration of 5 mg folic acid, 3.2±1.0 mg were detected in the urine; following iv administration, 3.8±1.1 mg were detected in the urine over the twelve hour period [78].

In another published study at higher doses, healthy adult volunteers were given either a single dose of 43 mg (*ca.* 1.4 μmol/kg-bw) or 216 mg (*ca.* 7.0 μmol/kg-bw) folic acid by both oral and iv administration separated by a two week washout. Plasma folic acid levels were determined immediately before and over a 24-hour period following treatment. The $t_{½}$ for the 43 mg dose was identical for oral and iv administration (2.5±0.2 hr and 2.4±0.1 hr, respectively). The AUC for the 43 mg dose taken orally was 3.1±0.33 mg•hr/ml and by iv was 5.1±0.36 mg•hr/ml. The $t_{½}$ for the 216 mg dose was 2.5±0.1 hr for oral administration and 3.1±0.1 hr for iv administration. The AUC for this dose taken orally was 6.2±0.8 mg•hr/ml and by iv was 36.7±2.9 mg•hr/ml. These results suggest that folic acid administered orally at the higher dose was not as efficiently absorbed as the lower dose [79].

Only trace amounts of folic acid are found in the urine following single oral doses of 0.1–0.2 mg (*ca.* 3.0–6.0 nmol/kg-bw) in healthy humans. When renal tubular reabsorption is exceeded with large oral doses, folate is excreted unchanged in the urine. After doses of 2.5–5 mg (*ca.* 0.08–0.2 μmol/kg-bw), about 50% of the dose is excreted in the urine; after a 15 mg dose (*ca.* 0.5 μmol/kg-bw), up to 90% is excreted in

the urine [76]. Folates secreted into the bile are reabsorbed through the intestine, resulting in enterohepatic circulation. Enterohepatic circulation is important for maintaining folate homeostasis and reutilizing folates released from dying erythrocytes [77].

CLINICAL EFFICACY: PHASE II/III STUDIES

The NCI, Chemoprevention branch has funded one Phase II and one Phase III ongoing clinical trials to investigate the effects of folic acid against colorectal adenomas and one completed Phase III trial in patients with CIN.

An NCI, Chemoprevention Branch-funded Phase II clinical trial with folic acid is in progress (Dr. Joel B. Mason, Tufts University, New England Medical Center). The study is designed to evaluate the efficacy of folic acid in patients with colorectal adenomas. Thirty patients will be administered 5 mg folic acid qd (*ca.* 0.2 μmol/kg-bw/day) orally for 24 months, then followed for 12 more months. The incidence of new polyps and regression of polyps are being assessed as well as the effects of folic acid treatment on DNA methylation and proliferation biomarkers. The NCI, Chemoprevention Branch is also funding a Phase III clinical trial to investigate the efficacy of folic acid on recurrence in patients with a history of colorectal adenomas (Dr. John A. Baron, Dartmouth College). Patients (300/arm) are administered 1 mg folic acid qd (*ca.* 0.03 μmol/kg-bw/day) orally with and without either 80 or 325 mg aspirin qd for three years, following a 3x2 factorial design. The study is in progress.

Published studies have shown folic acid treatment increased DNA methylation of rectal mucosa and decreased recurrence of colorectal adenomas. In a small published study, folic acid treatment resulted in increased DNA methylation of rectal mucosa, a proposed intermediate biomarker for colorectal cancer chemoprevention [11]. Patients were treated with 10 mg folic acid qd (*ca.* 0.3 μmol/kg-bw/day) or placebo orally for six months. Following treatment, patients receiving folic acid had significantly higher serum folate levels than before treatment (9.5±1.7 versus 23.7±0.2 ng/ml). Patients treated with folic acid also had significantly higher global methylation of DNA from rectal mucosa compared with placebo controls. Preliminary results from a small published study on patients who had colorectal adenomas removed by endoscopic polypectomy suggested that patients receiving 1 mg folic acid qd (*ca.* 0.03 μmol/kg-bw/day) orally for 24 months had a lower recurrence of colorectal adenomas than the placebo control group, although the difference did not reach statistical significance [80].

In a completed NCI-funded Phase III clinical trial, treatment with folic acid did not result in improvement of CIN lesions (Dr. Joseph Chu, University of Washington, Seattle). Patients (n=331) with koilocytic atypia, mild CIN or moderate CIN were randomized to receive either 5 mg folic acid (*ca.* 0.2 μmol/kg-bw/day) or placebo qd orally. After six months of treatment, there was no significant difference in the percent of patients showing histological improvement between the two groups, although the treated group had a three-fold higher serum folate concentration than the placebo group [53]. In contrast, in a published study, a group of eight oral contraceptive users who displayed megaloblastic changes in cervical epithelium received 10 mg folic acid qd (*ca.* 0.4 μmol/kg-bw/day) orally for three weeks. These patients showed significant reversal of cervical epithelial abnormalities when compared with their conditions before treatment [54]. The effect of folic acid treatment on serum folate levels was not determined in this study. In a small randomized, uncontrolled clinical trial controls, patients diagnosed with mild or moderate CIN received either 10 mg folic acid (*ca.* 0.4 μmol/kg-bw/day) or 10 mg ascorbic acid qd orally for three months. Patients receiving folic acid showed better biopsy scores compared with those receiving ascorbic acid and also an increase in RBC folate concentration from 161±20 ng/ml to 681±56 ng/ml after treatment [55]. A larger subsequent trial conducted by the same group over a six-month period did not reveal a significant difference between folic acid and ascorbic acid treatment, despite the fact that RBC folate levels for the treated group increased from 234±12 ng/ml to 753±50 ng/ml [56]. Since all these studies were performed on patients with established lesions, it is not known if folic acid treatment might be efficacious if given prior to the development of CIN.

Two intervention trials investigated the efficacy of combined treatment with folic acid and vitamin B_{12} on bronchial squamous metaplasia in smokers. In an NCI-funded trial, 73 men with a history of cigarette smoking who had metaplasia identified non-invasively in sputum samples were randomized to receive either placebo or 10 mg folic acid (*ca.* 0.3 μmol/kg-bw/day) plus 0.5 mg vitamin B_{12} qd orally for four

months. At the termination of the study, patients receiving the vitamin supplement had an RBC folate concentration of 1,185±56 ng/ml compared with 295±21 ng/ml at entry. After the four months of treatment, patients receiving folic acid and vitamin B_{12} showed an improvement in sputum cytology scores and a significant reduction in atypia compared with the placebo control group [57]. In a non NCI-funded study, patients with bronchial squamous metaplasia were treated with a combination of 10–20 mg folic acid (*ca.* 0.3–0.6 μmol/kg-bw/day) and 0.75 mg vitamin B_{12} qd orally or received no treatment for one year. The group receiving treatment showed a significant reduction in metaplasia in bronchial biopsies while the control group showed no improvement [58]. These studies cannot assess potential differential efficacies of the folic acid or vitamin B_{12} supplements, but based on the biochemical inter dependency of these compounds and their substantial human safety, there may be little scientific rationale for further investigation into the question of independent effects at this time.

PHARMACODYNAMICS

In the MCA-treated rat model, 15 mg folic acid (*ca.* 113 μmol/kg-bw) administered intramuscularly twice a week for 28 weeks significantly reduced the incidence of bronchial squamous metaplasia. This would be approximately equivalent to a human dose of 3.5 g folic acid twice a week, well above any dose previously tested in humans, although it has not been determined if folate absorption and metabolism are the same for rodents and humans. In addition, intramuscular injection of high doses of folic acid in humans has never been tested.

Epidemiological evidence has associated RBC folate concentrations below 140 ng/ml with increased risk of cervical cancer in women [39] and RBC folate concentrations less than 160 ng/ml are associated with an increased risk of colon cancer in men [36]. In one clinical trial, patients diagnosed with mild to moderate CIN receiving 10 mg folic acid qd (0.4 μmol/kg-bw/day) orally for three months showed an increase in RBC folate concentration from 161 to 681 ng/ml [55], a level well above that which is considered a deficiency risk factor for cancer. Additionally, patients with colorectal carcinomas and adenomas receiving 10 mg folic acid qd (0.3 μmol/kg-bw/day) orally for six months showed a significant reduction in global rectal mucosal DNA hypomethylation, an early event in the development of colorectal cancer, although it is unknown if lower doses might be equally effective [11].

PROPOSED STRATEGY FOR CLINICAL DEVELOPMENT

Drug Effect Measurement Issues

Folate status can be assessed by plasma or serum, RBC, or tissue levels. Determining the folate concentrations of plasma or RBCs is obviously much less invasive than measuring tissue folate concentrations, although the relevance of these surrogate measurements with regard to carcinogenesis is far from established. RBC folate levels are normally much higher than plasma levels and are less influenced by short term dietary fluctuations, thus providing a more accurate indication of global folate status [2]. Folic acid is essential for the synthesis of SAM; low levels of folic acid result in decreased tissue levels of SAM and increased levels of SAH [73]. When SAM levels are low, there is an increase in DNA hypomethylation [81]. Treatment with folic acid has been shown to increase tissue levels of SAM [73] and decrease global DNA hypomethylation [11]. Therefore, the ratio of SAM to SAH or the pattern and/or extent of DNA methylation in tissues of relevance are potential drug effect measurements for folic acid. In addition, low levels of serum and tissue folate are often associated with increased levels of serum homocysteine [3]. Homocysteine levels decrease in response to folic acid treatment; therefore, serum homocysteine levels are another potential drug effect measurement.

Safety Issues

Folic acid is generally regarded as safe in humans, though its pharmacologic administration is associated with a potential indirect hazard. Megaloblastic anemia is a common clinical presentation of individuals with either folic acid or vitamin B_{12} deficiency. Folic acid can effectively treat this anemia regardless of its cause. However, in addition to the anemia, vitamin B_{12} deficiency may result in a potentially irreversible neuropathy which is entirely preventable with appropriate vitamin replacement. Given this scenario, folic acid supplementation may inadvertently mask vitamin B_{12} deficiency by correcting the associated anemia, leaving the potential for harm from neuropathy. For this reason, any clinical trial must include preliminary and ongoing tests for this

deficiency [4,75]. Inclusion of up to 1 mg vitamin B_{12} in vitamin supplement capsules containing 400 μg folic acid has been recommended to protect vitamin B_{12} deficient-individuals [3]. Folic acid also inhibits absorption of the anticonvulsant drug phenytoin; therefore, patients receiving this medication should be excluded from clinical trials with folic acid. There is some evidence that folic acid may interfere with zinc absorption. If this does, indeed, pose a problem, it may be addressed by providing a zinc supplement.

Pharmacodynamics Issues

Oral doses of 10 mg folic acid qd (*ca.* 0.3 μmol/kg-bw/day) seem to be sufficient to increase RBC folate levels above 160 ng/ml. In epidemiological studies, lower serum concentrations have been associated with increased cancer risk [36,39]. Patient baseline RBC folate levels vary widely, which may have a significant impact on the efficacy of folic acid supplementation during cancer chemoprevention trials. RBC folate levels can be affected by the amount of folate in the diet, as well as individual differences in absorption and metabolism. Other factors, such as cigarette smoking and oral contraceptive use, are known to affect RBC folate levels [82,83]. Future NCI, Chemoprevention Branch trials could investigate the correlations between tissue-based assessments of folate status and metabolism, cancer risk, and potential surrogate measures of folate status and metabolism from easily attained samples such as serum or RBCs.

Regulatory Issues

Folic acid is approved for use in the US as a vitamin supplement, and folates are a natural component of a variety of foods. The US recommended dietary allowance is 200 μg/day (*ca.* 6.5 nmol/kg-bw/day) for adult males, 180 μg/day (*ca.* 6.4 nmol/kg-bw/day) for adult females and 400 μg/day (*ca.* 14.3 nmol/kg-bw/day) during pregnancy [1,2]. Preclinical studies have suggested that folic acid supplementation of folate-replete rats may increase the size and multiplicity of carcinogen-induced colon tumors in rats [63]. In order to support long clinical studies on patients with precancerous lesions, it may be necessary to conduct additional preclinical toxicity studies.

Intermediate Biomarker Issues

In a recently published study, global DNA methylation was used as an intermediate biomarker to evaluate the efficacy of folic acid against colorectal cancer [11]. It will be important to evaluate the effect of folic acid treatment on rectal mucosal DNA methylation patterns of specific genes, such as *ras*, *myc* and p53. Colorectal adenomas are premalignant lesions and have been used as histopathological intermediate biomarkers in chemoprevention trials. Particularly important are morphometric and cytometric changes in adenomas and surrounding normal-appearing mucosa. Proliferation-related biomarkers, such as ODC activity, PCNA and tyrosine kinase activity, are also useful intermediate biomarkers, but are probably most valuable when assessed in conjunction with apoptosis to attain a measure of integrated cellular population dynamics in tissues of relevance. A preclinical study demonstrated that folic acid was effective in modulating squamous metaplasia in the rat, a histological biomarker for lung cancer. Cytological evaluation of sputum samples to determine the degree of squamous cell metaplasia has also been used in clinical trials, though dysplastic assessments would be preferable. Cytologic sampling from readily available biological fluids, such as sputum, is attractive because it offers a non-invasive approach, but its ability to adequately represent the mucosa at neoplastic risk in either clinical or investigative settings remains unsubstantiated at present.

Supply and Formulation Issues

Folic acid is available in the US in oral tablet form. It can be purchased from a number of pharmaceutical companies, and so no supply problems are foreseen. It will be necessary to identify a suitable placebo.

Clinical Studies Issues

It is clear from both clinical trials and epidemiological studies that human baseline RBC folate levels vary widely, probably a consequence of the amount of folate in the diet as well as individual differences in absorption and metabolism. In future clinical trials, participant dietary folate intake should be regulated and patients stratified with respect to their baseline RBC folate levels. One criterion for including patients in a folic acid cancer chemoprevention trial could be RBC folate levels less than 160 ng/ml, since these patients might benefit most from folic acid supplementation.

The further development of folic acid as a chemopreventive agent against colorectal cancer awaits the outcome of the two NCI, Chemoprevention Branch-funded clinical trials in colon. Epidemiological studies and clinical trials suggest oral contraceptive use may decrease RBC and plasma folate levels, thus leading to an increased risk of developing CIN. Two early clinical trials reported that folic acid was efficacious against CIN in this patient population [54,55]. Future clinical trials investigating the efficacy of folic acid against CIN should include this patient population since they may benefit most from folic acid supplementation. Finally, folic acid in combination with vitamin B_{12} was shown to be effective against bronchial squamous metaplasia in smokers [57,58]. It has been suggested that cigarette smoke results in localized folic acid deficiency affecting primarily the bronchial epithelium through direct chemical inactivation [57,84]. Thus, smokers may represent another patient population who should be included in future clinical trials. Primary prevention prior to precancer should be explored as well as combinations with vitamin B_{12} and/or B_6 considering their interdependent biochemical roles.

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Table I. Clinical Trials of Folic Acid Sponsored/Funded by NCI, DCPC

Study No. Title (PI) Period of Performance IND No.	Cancer Target	Study Population No. Patients	Dose(s) Duration of Treatment	Endpoint(s)	Remarks
Phase II (Dose-titration, efficacy, intermediate biomarkers)					
UO1-CA-63812 Phase II Randomized, Double-blind, Placebo-controlled Study of High-dose Folic Acid for the Prevention of Colorectal Cancer in Patients with Resected Adenomatous Polyps (Dr. Joel B. Mason, Tufts University, New England Medical Center, ECOG) 7/94–6/97 Investigator IND	Colon	Colorectal adenomatous polyps; histologically proven 50 patients total (30 in folic acid arm and 20 in placebo arm)	Oral 5 mg folic acid or placebo qd for 24 months	Efficacy: Incidence and regression of polyps Intermediate biomarkers: Proliferation biomarkers, DNA methylation Safety	Grant funded in 1994 and is in progress Report: [85]
Phase III (Efficacy, intermediate biomarkers)					
PO1-CA-34847 Phase III Chemoprevention of Cervical Cancer with Folic Acid (Dr. Joseph Chu, University of Washington) 7/83-6/93 Investigator IND	Cervix	Koilocytic atypia, mild or moderate CIN 331 patients (168 treatment, 163 placebo)	Oral 5 mg folic acid or placebo qd for 6 months	Efficacy: Modulation of cervical dysplasia	Study complete. Results do not support the regression of early cervical epithelial abnormalities by 5 mg folic acid qd Median serum folate levels in the treatment arm were significantly higher than those in the placebo arm at three and six months Published report: [53]

Table I. Clinical Trials of Folic Acid Sponsored/Funded by NCI, DCPC *(continued)*

Study No. Title (PI) Period of Performance IND No.	Cancer Target	Study Population No. Patients	Dose(s) Duration of Treatment	Endpoint(s)	Remarks
Phase III (Efficacy, intermediate biomarkers) ***(continued)***					
RO1-CA-59005 Phase III Aspirin Prevention of Large Bowel Polyps. Randomized, Double Blind, Placebo-controlled Study on 2 Different Doses of Aspirin and Folic Acid for the Prevention of Recurrent Neoplastic Polyps of the Large Bowel in Patients with History of Adenoma (Dr. John A. Baron, Dartmouth College) 9/93–9/98 Investigator IND	Colon	Previous colorectal adenoma, 21–80 years of age	300 patients/arm (6 arms, total up to 1,800 patients)	Oral 1 mg folic acid qd (with and without 80 or 325 mg aspirin qd) for 3 years	Efficacy: Polyp recurrence (number and size) Safety Study in progress

FOLIC ACID DEVELOPMENT STATUS