RESEARCH STRATEGY

a) Background and Significance

Chlorophylls and chemoprevention: The Salmonella mutagenicity assay (Ames test) provided initial evidence for the anti-mutagenic activity of chlorophyll *a* (Chl *a*, Fig 1), Chl *b*, and the water-soluble sodium-copper salt, chlorophyllin (CHL) (Terwel and van der Hoeven 1985; Ong *et al*. 1986). Mechanistic work supported the formation of molecular complexes between planar aromatic carcinogens and the tetrapyrrole macrocycle in chlorins and porphyrins (Negishi *et al*. 1989; Dashwood and Guo 1992; Dashwood *et al*. 1996; Breinholt *et al*. 1995a), including with chlorin e6 (Ce6) and other *metal-free* analogs (Arimoto *et al.* 1993; Dashwood *et al*. 1996). CHL antitumor activity then was confirmed in trout, rats, and mice (Breinholt *et al*. 1995b; Guo *et al* 1995; Park and Surh 1996). Translating these findings to humans, carcinogen exposure biomarkers were reduced by CHL in a susceptible population (Egner *et al*. 2001), and carcinogen bioavailability was lowered by both CHL and **Chl *a* isolated from spinach** (Fig 2) (Jubert *et al.* 2009).

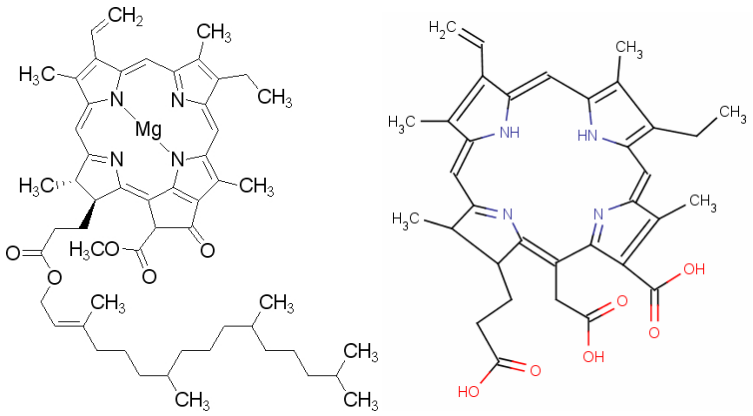

Fig 1 Chlorophyll *a* (Chl *a*) and copper-free chlorin e6 (Ce6).

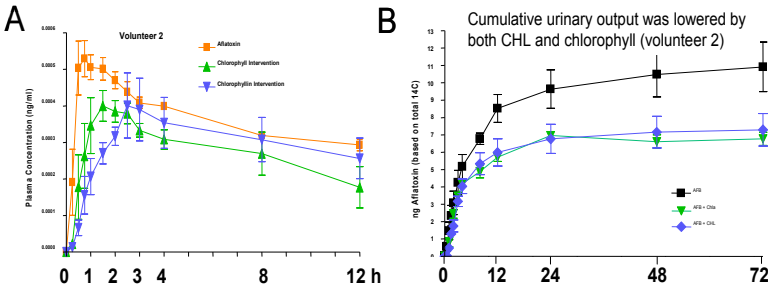

Fig 2 (A) Plasma AFB_1 levels were lowered by Chl or CHL; (B) less AFB_1 excreted after Chl or CHL treatment, Jubert *et al*. 2009.

Despite these promising findings, Cu-containing CHL and Cu alone also exhibited *promotional* activity in some studies (Nelson 1992; Xu *et al.* 2001; Blum *et al.* 2003). **This application focuses on natural Chl and the Cu-free analog Ce6**, and shifts from the 'blocking agent' concept (Wattenberg 1985) to cancer suppression via the **inhibition of ribonucleotide reductase (RNR)**.

CENTRAL HYPOTHESIS: Chlorophylls, chlorophyll-rich foods, and copper-free derivatives of chlorophyll will suppress tumorigenesis in the colon and lung, and will lack the promotional activity of CHL. Tumors accumulate chlorins and will be more susceptible to the inhibition of RNR activity and expression, resulting in selective S-phase arrest and apoptosis in cancer cells *versus* normal cells.

RNR as a mechanistic target in cancer prevention and treatment

RNR catalyzes the conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, which in turn are used for DNA synthesis. Because of its pivotal role in DNA synthesis, RNR has been recognized as a promising target for anticancer agents (Nordlund and Reichard 2006). We reported that human **colon cancer** cells treated with CHL undergo S-phase arrest prior to apoptosis induction (Díaz *et al.* 2003), and that this was associated with the **inhibition of DNA synthesis** and **loss of RNR enzyme activity and expression** (Chimploy *et al*. 2009). Inhibition of RNR *enzymatic activity* was revealed by direct addition of CHL or Chl *a* to whole cell lysates, whereas loss of *mRNA and protein* expression was established via qRT-PCR and Western blot studies (Chimploy *et al.* 2009). These

outcomes were observed in both $p53^{+/+}$ and $p53^{-/-}$ colon cancer cells (Chimploy *et al*. 2009), and in **Ce6-treated lung adenocarcinoma** cells (Fig 3). Loss of RNR subunit expression was in the relative order R2 > p53R2 >> R1. The small subunit **R2**, which peaks during S phase (Chabes *et al.* 2003), harbors a catalytically essential tyrosyl free radical that can be disrupted by anticancer agents such as **hydroxyurea** (HU) (Ababou *et al.* 2002; Chapman and Kinsella 2011; Madaan *et al.* 2012). Scavenging of tyrosyl radicals generated by myeloperoxidase in the presence of tyrosine and H_2O_2 has been reported for CHL *in vitro* (Kapiotis *et al.* 2005), but aim 1 will examine for the first time the actions of Chl, Ce6, and other chlorins towards the tyrosyl radical in R2 and p53R2. **p53R2** is an R2 analog induced by genotoxic stress (Tanaka *et al.* 2000), and can substitute for R2 in forming an active enzyme with the large subunit **R1**, which is expressed more or less uniformly throughout the cell cycle (Engström *et al.* 1985). Whereas R2 and p53R2 mRNA and protein expression were reduced by **μM levels** of CHL and Ce6 in colon and lung cancer cells, HU required mM concentrations for inhibition of enzymatic activity (Chimploy *et al.* 2009, Fig 3, and data not shown).

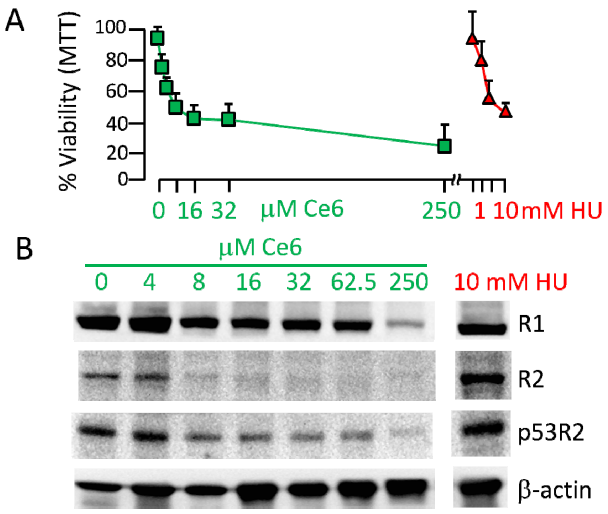

Fig 3 Human HTB174 lung adenocarcinoma cells treated with Ce6 or HU for 24 h. (A) MTT assay and (B) Western blots of RNR subunits and β-actin.

HU is a clinically used anticancer drug, but side effects include nausea, vomiting, anorexia, abnormal liver function, and myelosuppression (Madaan *et al.* 2012; Liebelt *et al.* 2007). As an alternative to HU and other R2 inducers (Ocean *et al.* 2011), **Ce6 accumulates in cancer cells to a greater extent than in normal cells**. This property has been exploited in photodynamic therapy (PDT), using Ce6, Chl *a*, pheophorbide *a,* and other tetrapyrroles (Chin *et al.* 2007; Rapozzi *et al.* 2010; Ali-Seyed *et al.* 2011; Srivatsan *et al.* 2011; Xodo *et al.* 2012). This application considers such compounds as RNR inhibitors, with efficacy beyond the normal scope of PDT. Moreover, the 'natural product' approach to inhibition of R2 and p53R2 might prove more effective than RNAi (Davis *et al.* 2012).

b) Innovation

- Ce6 and related compounds are currently used in PDT, but their anticancer properties have not been thoroughly examined in a non-PDT context, and specifically as inhibitors of RNR.
- Compared with RNAi/nanoparticle delivery, Chl and Ce6 might offer a more practical therapeutic approach due to targeting both R2 and p53R2, via the inhibition of enzymatic activity and downregulation of mRNA and protein expression, and using lower concentrations than HU.
- Novel mouse models overexpressing R2 and p53R2 will be employed for the first time in tumor suppression studies, focusing on systemic versus GI tract efficacies of spinach, Chl, and Ce6.
- Translational studies will take advantage of an ongoing screening colonoscopy trial to ascertain possible changes in RNR activity and expression in the context of the central hypothesis.
- Preclinical and clinical tissue samples will be examined for Chl metabolites via LC-MS/MS.

c) Approach

Three specific aims are proposed that coordinate and integrate mechanistic studies (Aim 1), preclinical experiments (Aim 2), and proof-of-concept translational studies in humans (Aim 3).

Aim 1 MECHANISMS – **aim 1a** tests the hypothesis that Chl, Ce6, and other chlorins disrupt the tyrosyl radical in RNR, using electron paramagnetic resonance (EPR) spectroscopy; **aim 1b** applies *in silico* modeling of Chl, Ce6, and other chlorins with RNR subunits R1, R2, and p53R2; **aim 1c** will examine the kinetics of RNR inhibition by Chl, Ce6, and related chlorins *in vitro*; **aim 1d** uses chromatin immunoprecipitation (ChIP) assays to test the hypothesis that, in colon and lung cancer cells, downregulation of *R2* and *p53R2* mRNA expression is associated with changes in transcription factors and binding partners on the corresponding gene promoters; **aim 1e** will examine mechanisms affecting *R2* and *p53R2* mRNA stability, including the actions of miRNAs; **aim 1f** will assess in both human normal and cancer cell lines (colon, lung) cell cycle arrest and apoptosis following knockdown and overexpression of R2 and p53R2.

Rationale – Because of its pivotal role in DNA synthesis, RNR has been recognized as a promising target for anticancer agents. HU and other tyrosyl radical scavengers typically induce R2 expression, whereas recent clinical trials with RNAi and nanoparticle delivery sought to downregulate R2. A more straightforward approach, with reduced side effects, might be to employ dietary agents or their analogs to simultaneously target both the enzymatic activity and expression levels of R2 and p53R2. Preliminary work identified CHL and Ce6 acting in this fashion in human colon and lung cancer cells. To provide further mechanistic insights, EPR, molecular modeling, and enzyme kinetic studies will clarify the nature of the enzyme inhibitory activity, whereas stability and turnover mechanisms will be assessed via ChIP analyses of the *RRM2* and *RRM2B* promoters, plus mRNA and protein changes.

Methods

Statistics Unless stated otherwise, each experiment will be repeated three times, with data shown as mean±SD. Student's *t*-test will be used for paired comparisons, and ANOVA for group comparisons. In published manuscripts figures will indicate **P*<0.05, ***P*<0.01, ****P*<0.001, or *P*>0.05, as appropriate.
Cell culture and test agents HCT116, CCD841, HTB174, HBEpiC, and other cell lines from ATCC (Manassass, VA) or ScienCell Res Labs (Carlsbad, CA) will be authenticated before use (Parasramka *et al.* 2012a). Based on preliminary experiments, cells will be seeded at the appropriate density in the recommended media and treated with test agents in order to examine time-dependent changes in target mRNA and protein expression. Chl *a*, Chl *b*, Ce6, and other tetrapyrroles (>95% purity) will be as reported (Chimploy *et al.* 2009; Jubert *et al.* 2009; Jubert and Bailey 2007; Dashwood *et al.* 1996). Stock solutions will be prepared/diluted before each experiment (conducted under subdued lighting).
Knockdown and overexpression of R2 and p53R2 will follow reported protocols (Lin *et al.* 2004; Yoshida *et al.* 2006; Rajendran *et al.* 2011; Wang *et al.* 2011; Parasramka *et al.* 2012a).
mRNA analyses will be done by qRT-PCR, using the basic methodologies reported (Wang *et al.* 2013; Chimploy *et al.* 2009). Initial focus will be on *R2* and *p53R2,* normalized to *GAPDH*.
miRNA analyses will follow reported protocols (Parasramka *et al.* 2012a, 2012b). Computer-based prediction and validation of miRNAs targeting *R2* and *p53R2* mRNAs will be followed by Metacore pathway analyses and mechanistic studies, including silencing and overexpression experiments.
Immunoblotting of cytoplasmic, nuclear, and whole cell extracts will follow reported methodologies (Wang *et al.* 2013; Rajendran *et al.* 2011; Chimploy *et al.* 2009). In addition to R1, R2, and p53R2, targets of interest will be key cell cycle regulators and apoptosis inducers (*e.g.*, p21, p53, Bcl-2 family members, active caspases, and cleaved PARP). Equal protein loading will be confirmed by Amido Black staining, β-actin, or histone H1. After incubation with primary and then secondary antibodies, immunoreactive bands will be visualized/quantified via chemiluminescence (Alpha Innotech imager).
Flow cytometry Adherent and non-adherent cells will be collected at selected time points after treatment, fixed in 70% ethanol, and stored at 4°C for at least 48 h. Fixed cells will be washed with PBS and resuspended in propidium iodide (PI)/Triton X-100 staining solution containing RNaseA. Samples will be incubated in the dark for 30 min before cell cycle analysis (as in Rajendran *et al.* 2011).

ChIP assays will follow the basic methodologies reported (Nian *et al.* 2009; 2008). At selected times after treatment with test agents (Chl, Ce6, other chlorins), the promoter and other regions of *RRM2* and *RRM2B*, plus a negative control gene (*ACTB*), will be interrogated for changes in histone marks, chromatin modifiers, and transcription factors such as E2F4 (Chimploy *et al.* 2009).
RNR activity will be determined *in vitro* and *in vivo* as reported (Chimploy *et al.* 2009). Dr. Christopher K. Mathews will assist in these experiments, and in the training of lab personnel (see letter of support).
EPR studies will be performed by Dr. Astrid Gräslund (Stockholm University, see letter), using reported methodologies (Heffeter *et al.* 2009). In brief, quenching of the tyrosyl radical in R2 by Chl *a*, Ce6, and other chlorins will be monitored kinetically in the presence of R2 protein expressed in Rosetta 2(DE3)pLys bacteria, as described (Mann *et al.* 1991). The effects of reducing agents and/or oxygen or peroxide will be investigated. EPR spectra will be recorded on a Bruker ESP 300 X-band (9.5 GHz) spectrometer with an Oxford Instruments ESR9 helium cryostat (conditions typically: 40 K, 3.2 mW microwave power). The tyrosyl radical concentration will be determined by double integration of EPR spectra recorded at non-saturating microwave power levels, and compared with a standard solution of 1 mM $CuSO_4$ in 50 mM EDTA. The calculated radical concentration will be normalized and expressed in percent. Experiments will be repeated 5 times, with an estimate of uncertainty of each measurement of about 5%. Subsequent experiments will use the p53R2 protein with the same test agents.
Molecular modeling will be performed by Dr. W.H. Bisson (see letter), a coauthor on the PI's recent publications (Nian *et al.* 2009; Lee *et al.* 2009; Larsen *et al.* 2009; Larsen *et al.* 2010). Coordinates of human R1, R2, and p53R2 and mouse R2 will be taken from available crystal structures in the Protein Data Bank (PDB 2WGH, 2UW2, 3HF1, 1XSM, respectively). The mouse R1 and p53R2 models will be built based upon the corresponding human protein crystal structure as the 3D template using Molsoft ICM v3.7-1d. Each model then will be energetically refined in the internal co-ordinate space, with docking represented by: (i) van der Waals potential for a H-atom probe; (ii) van der Waals potential for a heavy-atom probe; (iii) optimized electrostatic term; (iv) hydrophobic terms; and (v) loan-pair-based potential, which reflects directional preferences in hydrogen bonding. The energy terms will be based on the all-atom vacuum force field ECEPP/3 with appended terms from the Merck Molecular Force Field to account for solvation free energy and entropic contribution. Modified inter-molecular terms such as soft van der Waals and H-bonding, as well as a hydrophobic term, will be included. Conformational sampling will be based on the biased probability Monte Carlo procedure, with full local minimization after each randomization step. In the screening procedure, ligand scoring will be optimized to obtain maximal separation between bound and unbound species. Each test compound (Chl *a*, Chl *b*, Ce6, and other chlorins) will be assigned a score according to fit with the active site pocket, or other potential areas of interaction, accounting for electrostatic, hydrophobic, and entropy parameters.

Anticipated results, potential pitfalls, alternative strategies Based on the central hypothesis, findings for CHL (Díaz *et al.* 2003; Chimploy *et al.* 2009) will be recapitulated in both colon and lung cancer cells by Chl, Ce6, and other chlorins with respect to: (i) E2F4 induction, (ii) RNR downregulation (R2>p53R2>>R1), (iii) inhibition of DNA synthesis, and (iv) S-phase arrest. EPR, molecular docking, and enzyme inhibition studies will identify the most effective tetrapyrroles for RNR inhibition, which will be examined mechanistically in terms of disruption of the tyrosyl radical, non-competitive inhibition, and allosteric changes affecting sub-unit interactions. *In silico* modeling suggested the possibility for Ce6 interactions affecting the dimer interface of R1 subunits (Fig 4A, overleaf), an allosteric site in R2 (Fig 4B, human and mouse residues in green and purple, respectively), and the active site in p53R2 (Figs 4C-E; Fe atoms, blue spheres; tyrosyl radical site, green; HU docked, purple). The proposed molecular modeling of chlorins in aim 1b will examine these, and other potential interactions, in detail.

Ectopic overexpression of R2, p53R, or R2+p53R2 will be tested as a means of rescuing Chl- or Ce6-treated cells, whereas R2+p53R2 double knockdown should mimic effects of the test agents on S-phase arrest and apoptosis. In prior reports, ectopic expression of p52R2 alone did not compensate

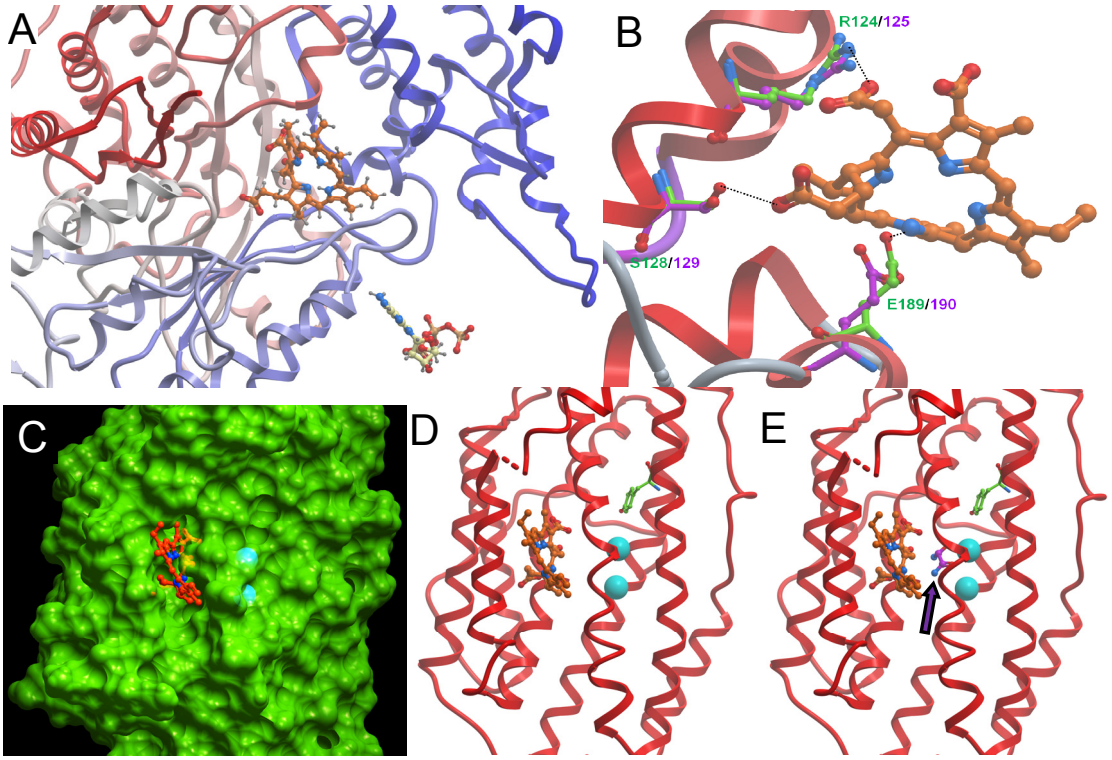

Fig 4 Ce6 docked with RNR subunits (A) R1, (B) R2, (C-E) p53R2; arrow, HU position (Molsoft ICM v3.5 1p)

for the decreased levels of R2 brought about by *R2*-siRNA, whereas p53R2 knockdown attenuated the DNA repair response induced by short-chain fatty acids (Lin *et al.* 2004; Yoshida *et al.* 2006).

Another anticipated result is that Chl, Ce6, and other chlorins will have **less effect in non-transformed cells than in cancer cells**. In pilot experiments, concentrations of Ce6 that reduced R2 and p53R2 protein expression in HTB174 lung cancer cells (Fig 3) and in HCT116 colon cancer cells (Fig 5) had no such effect in CCD841 cells. Also, Ce6-treated CCD841 cells had no increased apoptosis based on published morphological criteria (Díaz *et al.* 2003) and studies of cytochrome *c* release, PARP cleavage, and caspase activation (data not shown).

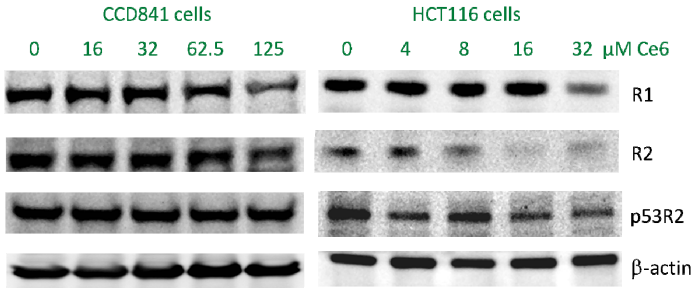

Fig 5 Ce6 reduced R1, R2, and p53R2 protein expression at lower concentrations in HCT116 colon cancer cells than in CCD841 non-transformed colonic epithelial cells (24 h). Aim 1 also will compare cancer and normal lung epithelial cells.

Chl, Ce6, and related chlorins will be titrated carefully in each cell line (starting nominally at 10 μM) before detailed ChIP, qRT-PCR, and Western blotting studies. Focusing on *transcriptional* changes that affect RNR activity, time-course data will identify early alterations on the *RRM2* and *RRM2B* gene promoters (within 1-3 h), followed by reduced R2 and p53R2 mRNA (3-6 h) and protein expression (6-24 h). ChIP analyses will show: (i) loss of histone marks associated with gene activation (e.g., H3K4me3, H3K9ac), (ii) gain of histone marks associated with gene repression (e.g., H3K9me3, H3K27me3), (iii) the (de)recruitment of chromatin remodeling complexes (e.g., PcG, Trx) (Schwartz *et al.* 2010; Balasubramanian *et al.* 2011), and (iv) binding of E2F4 (Chimploy *et al.* 2009) and other 'inhibitory' transcription factors and their corepressors, which will be verified in co-IP assays.

Alternatively, qRT-PCR and Western blot data might show increased *instability* and *turnover* of R2 or p53R2 mRNAs or proteins. This avenue would be pursued using reagents such as cycloheximide and actinomycin D, as in recent studies of HDAC turnover (Rajendran *et al.* 2011). Increased *mRNA turnover* or *stalled transcription* in response to the test agents would lead to miRNA overexpression and knockdown experiments, as reported (Parasramka *et al.* 2012a,b). Using TargetScan, miRanda, mirSVR, and other computational tools we identified 3'-UTR regions in *RRM2* and *RRM2B* mRNAs as potential targets of **let-7 family members**, the significance of which is explained in Aim 2. Experimental verification of miRNAs followed by target validation will be performed (as in Parasramka *et al.* 2012a,b). Protein stability/turnover mechanisms would examine post-translational modifications to R2 and p53R2, transcription factors (E2F4, E2F1), miRNA biogenesis intermediates (e.g. Drosha, Dicer, Ago2, RISC), and key regulators of the cell cycle. If Chl, Ce6, and other chlorins affect both gene transcription and mRNA/protein stability, this would further support the efficient downregulation of R2 and p53R2 in colon and lung cancer cells. It is possible that certain chlorins will act directly on targets besides RNR, causing G1 or G2/M arrest, rather than S phase arrest. This would be beyond the scope of the stated central hypothesis, but nonetheless of interest for possible anticancer therapy.

Aim 2 PRECLINICAL – **aim 2a** uses novel RNR overexpressing mice to test the hypothesis that orally administered Chl, Ce6, and spinach will suppress spontaneous tumor formation in the lung; **aim 2b** will administer a colon carcinogen to RNR overexpressing mice in order to assess suppression versus possible promotional activities of Chl, Ce6, and dietary spinach; **aim 2c** will examine RNR activity and expression in target and non-target tissues of mice, plus molecular endpoints of cell proliferation and apoptosis (from aim 1); **aim 2d** tests the hypothesis that dNTP pool imbalances in the presence of oxidative stress (lung) or genotoxic stress (colon) predispose to increased tumor formation; **aim 2e** applies LC-MS/MS to examine Chl and Ce6 fate in the plasma, urine, and tissues of mice.

Rationale – Overexpression of *Rrm2* and *p53R2*, but not *Rrm1*, was reported to induce lung tumor formation in mice (Xu *et al.* 2008). These genetic models were obtained from Dr. Robert S. Weiss (Cornell University) and bred at the Linus Pauling Institute. Aim 2a first determines whether oral administration of Chl, Ce6, and spinach achieves sufficiently high systemic levels to inhibit spontaneous tumors in the lung. In aim 2b, RNR overexpressing mice will be treated with 1,2-dimethylhydrazine (DMH), and in the *post*-initiation phase test agents will be examined for tumor suppressing versus possible promotional activities. Mechanistic sub-aims (2c and 2d) focus on changes in RNR activity and expression in line with the central hypothesis, plus the role of dNTP pool imbalances in target and non-target tissues. Finally, due to the paucity of reliable bioavailability data *in vivo*, state-of-the-art LC-MS/MS analyses will be undertaken in mice treated with stable isotope labeled Chl and Ce6 (aim 2e).

Methods

Tumor suppression (lung) Mice will be assigned randomly to groups according to genotype and test agent (Fig 6). Ce6, Chl, and 10% freeze-dried spinach will be administered in the diet from weaning to the end of the study. Controls will receive AIN93 diet alone. Previous work showed that 10% dietary spinach was well tolerated in mice (Castro *et al.* 2009). Ce6 and Chl doses will be matched to the total chlorin content in 10% spinach diet, following the general approach reported (Pratt *et al.* 2007). Using counter-current chromatography (Jubert and Bailey, 2007), 30 g freeze-dried spinach yielded 300 mg Chl *a* and 100 mg Chl *b*. Mice will receive Chl *a*, unless mechanistic studies in aim 1 provide a rationale for Chl *b* as being more effective against RNR. QA/QC analyses will be performed routinely on batches of spinach and test diet. Adjustments will be made as necessary to maintain the chlorin content as consistent as possible throughout the study. To minimize Chl degradation, diet sufficient for one week will be stored in the dark at 4^{o}C in sealed black bags, and food will be placed in the cages after lights-out (as in Blum *et al.* 2003). As a positive control for RNR inhibition, HU will be given orally at the nominal concentration of 30 mg/kg body weight (Sampson *et al.* 2010). When the study is terminated at

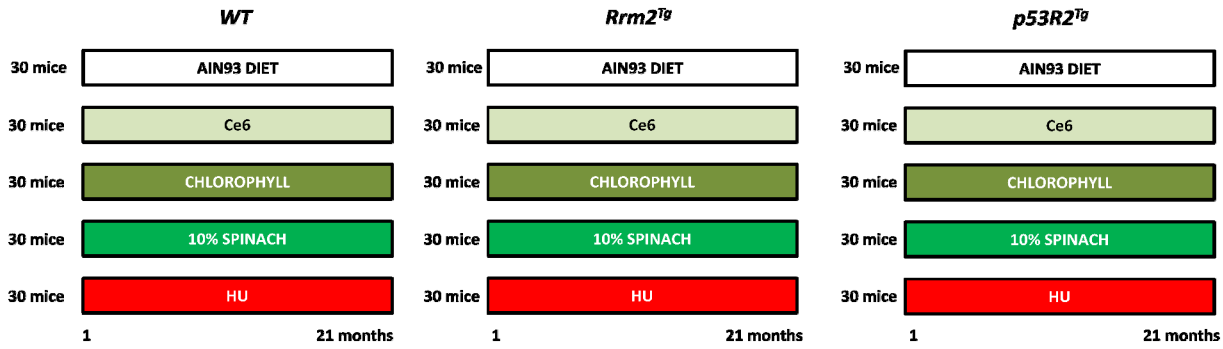

Fig 6 Suppression of spontaneous lung tumors in wild type (*WT*) mice, and in transgenic mice overexpressing RNR subunits R2 (*Rrm2*^{Tg}) or p53R2 (*p53R2*^{Tg}). Mice (n=30) will be fed 10% spinach in the diet, or Chl and Ce6 at the equivalent chlorin content. HU, hydroxyurea.

21 months, a complete necropsy exam will be performed. Lung tumors and other lesions in target and non-target tissues will be enumerated and examined by a trained histopathologist (see letter, Dr. Christiane V. Löhr). Portions of tumor and normal tissue will be frozen at -80^{o}C for molecular analyses.

Statistics Comparisons will be made between control and test treatments in each of the genotypes. A tumor incidence of 72-74% is expected in *Rrm2*^{Tg} and *p53R2*^{Tg} mice (Xu *et al.* 2008), with ~50% having multiple lung neoplasms. Assuming Chi-square tests can be used (as in Xu *et al.* 2008), then 30 mice/ group will have ~80% power to detect a reduction from 74% to 39% (a reduction of 35%) with a 0.05 significance level. Xu *et al.* (2008) reported average (and SD) lung neoplasm size (mm) of 6.68±4.22 and 4.26±3.44 in *Rrm2*^{Tg} and *p53R2*^{Tg} mice, respectively. Assuming the larger coefficient of variation (SD/mean), 30 mice/group will have 80% power to detect a 41% reduction in tumor size using a two-sided *t*-test with a 0.05 significance level. If necessary, non-parametric methods (e.g., Kruskal-Wallis and Wilcoxon rank tests) will be used – see letter from statistician Dr. C. Pereira.

Tumor suppression (colon) Based on the results from aim 2a, *Rrm2*^{Tg} or *p53R2*^{Tg} mice and *WT* mice will be treated with DMH, followed by the test compounds (Fig 7). A dose-response is included for Ce6, to parallel prior studies with CHL that revealed tumor promotion *versus* suppression in the colon (Xu *et al.* 2001). The protocol calls for administration of 20 mg DMH/kg body wt once per week for 5 weeks, a one week interval with no DMH injection, and then test agent treatment until the end of the study at 35 weeks. After a complete necropsy exam, tumors and other lesions in target and non-target tissues will be enumerated and examined by Dr. Löhr. Portions of colon tumor and adjacent normal-looking colonic mucosa will be frozen at -80^{o}C for molecular analyses.

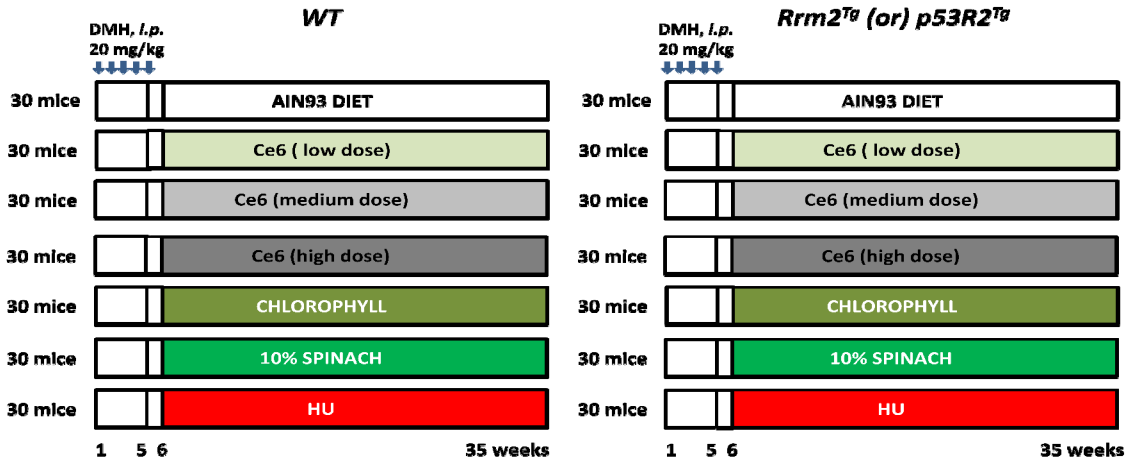

Fig 7 Suppression of colon tumors in *WT* and RNR overexpressing mice. Mice (n=30/group) will be treated with DMH during the first 5 wks and, after a 1 wk interval, test agents will be given as in aim 2a.

Statistics Comparisons between control and test treatments will follow the basic approach outlined in Aim 2a. In *WT* mice, Xu *et al.* (2001) observed a multiplicity (colon tumors/tumor bearing animal) of 2.7±1.05 (CV=SD/mean=0.39) and an incidence of 87%. Assuming a Chi-square test can be used, then 30 mice/group will have 80% power to detect a reduction in incidence from 87% to 55% (a reduction of 32%) with a 0.05 significance level. Although there will be low power to detect an increase in tumor incidence from the already high levels expected in *WT* (87%), multiplicity can be used for detecting an increase in tumor response. When there is a high incidence in both treatment groups (≥87% giving ≥26 tumor bearing animals per group), and assuming roughly log normal data with the above observed CV=0.39, there will be ≥90% power to detect a fold change in means (expected ratio) multiplicity of 1.42 using a two-sided t-test with a 0.05 significance level. When the observed distribution of data requires a non-parametric analysis (Wilcoxon test), the power is expected to be slightly lower. If RNR^{Tg} mice are more sensitive than *WT* to DMH, then time of mortality due to colon tumors may become an endpoint of interest, which could be analyzed with log rank and Cox proportional hazard regression

Molecular endpoints Changes in RNR subunit expression and localization will be given first priority, using qRT-PCR, Western blotting, and immunohistochemistry approaches (Wang *et al.* 2013; 2011; 2008; 2006). Markers of cell proliferation and apoptosis will be examined *in situ* (BrdU and cleaved caspase-3 labeling indices). It is anticipated that key molecular targets of interest will include E2F transcription factors, regulators of S-phase transition, and Bcl-2 family members. Depending on results from aim 1, profiling of miRNA signatures will be undertaken in tumors and matched normal tissue (Parasramka *et al.* 2012a), as well as ChIP assays of relevant target genes.

dNTP pools The basic approach to examining dNTP pool imbalances in target and non-target tissues of *WT*, $Rrm2^{Tg}$ and $p53R2^{Tg}$ mice will be as reported (Song *et al.* 2003; Koç *et al.* 2004; Wheeler and Mathews 2011). Dr. Mathews will provide hands-on assistance/training of lab personnel (see letter).

LC-MS/MS analyses O^{18}-labeled Chl *a* and Ce6 will be custom synthesized, followed by LC-MS/MS analyses (Morishita and Tamiaki, 2003; Porra *et al.* 1998). Initially, urine, plasma, and tissue homogenates of lung and colonic mucosa from control (untreated) mice will be spiked *ex vivo* with O^{18}-labeled test compounds in order to optimize LC-MS/MS conditions (see letter, Dr. Claudia Maier). Subsequently, mice will be treated by single oral gavage with O^{18}-labeled test compound, and at 1, 3, 6, 24 and 48 h urine, plasma, and tissues will be collected (n=3 per timepoint) for LC-MS/MS analysis. Chl *a* and Ce6 doses will be matched with the estimated daily intake determined under aims 2a and 2b.

Anticipated results, potential pitfalls, alternative strategies Based on the central hypothesis and preliminary data, it is anticipated that mechanistic findings in aim 1 will be corroborated in aim 2 with respect to (i) downregulation of RNR (R2>p53R2>>R1), (ii) inhibition of DNA synthesis, and (iii) cell cycle arrest/apoptosis induction in target tissues of mice treated with Chl, Ce6, and dietary spinach, resulting in tumor suppression. In studies lasting 21 months versus 35 weeks (Figs 6 and 7), inhibition of tumorigenesis might be greater in lung than colon. The reverse scenario, however, might reflect lower systemic distribution of test agent or metabolites, and relatively high levels in the gut after oral administration. We would seek to verify these outcomes in the bioavailability studies, including with stable isotope-labeled compounds given shortly before euthanasia, where accumulation in tumors would be expected. Once the specific metabolites of Chl and Ce6 have been ascertained with stable isotope-labeled compounds, subsequent LC-MS/MS analyses will not depend on labeled material to track these intermediates in tissues of mice given Chl, Ce6, and spinach.

The precise molecular basis for lung tumor formation in $Rrm2^{Tg}$ and $p53R2^{Tg}$ mice, but not in $Rrm1^{Tg}$ mice, is unknown. Two possibilities were discussed by Xu *et al.* (2008). Increased R2 or p53R2 levels might cause dNTP pool imbalances that impair replication fidelity and trigger mutations in growth regulatory genes. In this scenario, suppression of spontaneous lung tumors by Chl, Ce6, and dietary spinach might be associated with normalization of dNTP pools, and fewer activating mutations in oncogenes (e.g., *K-ras*) or inactivating mutations in tumor suppressors (e.g. *p53*). Overexpression of R1 is not expected to affect dNTP pools because this protein is normally present in excess throughout

the cell cycle (Engström *et al.* 1985). Proportional expansion of dNTP pools by RNR overexpression is mutagenic, as shown in *E. coli* (Wheeler *et al.* 2005), probably because a general increase in dNTP concentrations at replication sites preferentially increases rates of DNA chain extension from mismatched nucleotides at 3' termini, while chain extension rates from matched termini remain unchanged because they are already at Vmax. Hence, the ratio of incorrect to correct nucleotide incorporation increases (Mathews, 2006), leading to substitution mutations.

Alternatively, lung tumor formation might be independent of nucleotide metabolism. Xu *et al.* (2008) noted that each RNR catalytic cycle generates a tyrosyl free radical in the small subunit, and thus overexpression of R2 or p53R2 might lead to increased radical generation and formation of reactive oxygen species, especially in the high O_2 environment of the lung. The associated chronic oxidative stress might be targeted effectively by Chl, Ce6, and spinach 'antioxidants', as discussed by Chimploy *et al.* (2009). In this context, the mechanistic insights from EPR, molecular docking, and enzyme inhibition studies (Aim 1) will be valuable, especially if they support direct scavenging of the tyrosyl radical by Chl and Ce6 versus, say, noncompetitive inhibition with respect to the rNDP substrate. In tissues other than lung, genotoxic stress rather than oxidative stress might be important. In a pilot study, mice were injected *i.p.* with DMH (20 mg/kg body wt) and by 12 wks *p53R2*^{Tg} mice had significantly more aberrant crypts than *WT* or *Rrm1*^{Tg} mice treated under the same conditions (Fig 8). Based on these findings, *Rrm2*^{Tg} or *p53R2*^{Tg} mice might have significantly higher colon tumor multiplicity than *WT*, and a greater 'dynamic range' for tumor suppression by Chl, Ce6, and spinach.

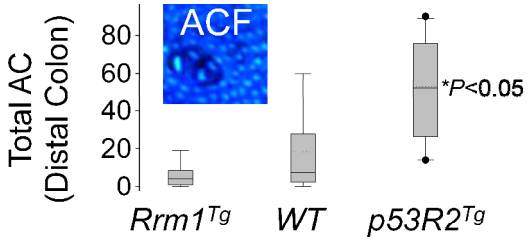

Fig 8 Mice overexpressing p53R2 are more susceptible to DMH-induced colonic aberrant crypt foci at 12 weeks (n=10 mice/group). WM Dashwood *et al.* unpublished data.

Dietary spinach recently was shown to suppress multi-organ tumorigenesis in the rat (Parasramka *et al.* 2012b). In an unbiased screen of 679 miRNAs, members of the let-7 family were highly dysregulated in colon tumors, and these were normalized by spinach treatment. Computational modeling and target validation identified c-Myc and miRNA-binding proteins Lin28A/Lin28B as key players, along with pluripotency factors Sox2, Nanog, and Oct-3/4. As noted above, the 3'-UTR regions in *RRM2* and *RRM2B* mRNAs were identified as potential targets of let-7, as were the corresponding murine transcripts. Thus, one very interesting avenue of research will be to integrate the mechanistic studies from aim 1 with miRNA profiling and target validation *in vivo*, in mice treated with Chl, Ce6, and spinach (in aim 2). The hypothesis under test is that spinach, Chl, and Ce6, in normalizing let-7 expression, facilitate miRNA binding to *Mmr2* and *p53R2* mRNAs and enhanced turnover/instability of the corresponding transcripts. Changes at the gene expression level would parallel mechanistic work under aim 1, including ChIP analyses of the corresponding gene promoters.

An alternative outcome, of course, is that other factors in spinach besides Chl modify miRNA signatures or, more broadly, RNR expression and activity. The Food Composition Laboratory in the Linus Pauling Institute will provide phytochemical profiling, for follow-up studies and potential new leads in the context of the central hypothesis (see letter from R. Durst).

Aim 3 TRANSLATIONAL – **aim 3a** builds upon an ongoing screening colonoscopy trial that administers a food frequency questionnaire to test the hypothesis that high intake of green/leafy foods is associated with altered RNR expression in polyps compared with normal colonic mucosa; **aim 3b** examines the corresponding peripheral blood mononuclear cells (PBMCs) to test whether RNR expression levels (*R2*, *p53R2*) can inform on the changes detected in polyps and normal colon biopsies; **aim 3c** builds upon LC-MS/MS studies in aim 2e to characterize Chl metabolites in plasma and tissue biopsies as potential biomarkers of intake from whole foods, such as spinach.

Rationale – An IRB-approved screening colonoscopy trial under P01 CA090890 ("Comparative Mechanisms of Cancer Chemoprevention", R.H. Dashwood, PD) is in progress, in conjunction with Oregon Health & Science University (OHSU, Portland, OR). This small proof-of-concept 'Phase 0' study provides an opportunity to translate mechanistic and preclinical work in aims 1 and 2 to humans.

Methods

Screening colonoscopy trial From the funded P01 (with key changes shown in Calibri font): "In patients presenting for screening colonoscopy, administer a validated food frequency questionnaire (FFQ), and assess RNR status in colon tissue biopsies (aim 3A) and in circulating PBMCs (aim 3B). In aim 3C, assess Chl and its metabolites in human plasma samples and in colon tissue biopsies using LC-MS/MS approaches developed under aim 2. The number of subjects approved for recruitment is 150, but this could be expanded to 200 in order to provide additional tissue samples for screening of RNR status. Recruitment and data collection will be implemented through the Oregon Clinical and Translational Research Institute (OCTRI) and the OHSU Cancer Institute".

Recruitment, informed consent, clinic visit The table below summarizes the Schedule of Events. After obtaining informed consent and administering the FFQ, blood will be drawn for pre-procedure tests (blood count, coagulation, metabolic panel), as well as for study specimens.

Schedule of Events	Telephone Screening	Clinic Visit	Telephone Interviews (3)	Colonoscopy Visit	Telephone Contact
		1 wk before colonoscopy	Random dates over 2-3 wks	Day of colonoscopy	3-5 d post colonoscopy
Entry Criteria	x				
Informed Consent		x			
Screening Blood Sample		x			
Blood study samples (2x10 mL)		x			
Demographics, Medical History		x			
FFQ (spinach/baby spinach line items)		x			
24-Hour Diet Recalls			x		
Collection of colon tissue				x	
Clinical findings				x	
Change in diet/medication assessment				x	
Adverse Event Assessment					x

Colonoscopies will be performed at OHSU or the Portland VA Medical Center, with records kept of the presence/location of polyps and clinical outcome: 1) no neoplasia; 2) neoplasia (not advanced); 3) advanced neoplasia (adenoma >10 mm, villous, high-grade dysplasia, invasive cancer). With prior consent, biopsies of normal-appearing tissue also will be taken (proximal and rectal colon, two in <u>each</u> location). To be included in the data analysis, participants must have a complete exam to the cecum.

Diet Assessment A validated FFQ will be administered by trained staff, capturing data on the number of servings, serving size, cooking method, and intake of raw and cooked vegetables, including oriental mixed dishes and condiments. The FFQ is completed in 20 min, and the data scanned and analyzed by the Diet, Behavioral & Quality of Life Assessment Center at the University of Arizona (Thomson *et al.* 2007). The FFQ is focused mainly on crucifers, which includes several green leafy vegetables; spinach and baby spinach will be added as specific line items. Three 24-h recalls will capture additional information on food intake, including spinach and other green leafy vegetables, giving information on *overall* diet patterns. Volunteers will be asked about changes in diet/medications between the clinic visit and colonoscopy.

Molecular biomarkers Colon tissue biopsy specimens and PBMCs will be subjected to qRT-PCR and immunoblot analyses of R2, p53R2, and R1, using methodologies outlined in aim 1. Tissue microarrays (TMAs), as in Wang *et al*. (2013, 2011), will be subjected to immunohistochemical analyses of R2, p53R2, and R1. The percentage of cells staining positive for each RNR subunit will be recorded in a given field of vision, as will the average staining intensity.

Statistical considerations Associations will be evaluated between green leafy vegetable intake, as assessed by the FFQ, and the following biomarkers: R2, p53R2, and R1 mRNA and protein expression in PBMCs and colon biopsies. Dietary intake will be treated as a continuous variable (servings/day). qRT-PCR data are continuous variables, whereas immunohistochemistry studies will be scored 0,1,2,3. For predictors with a normal distribution in a linear regression setting, a sample size of 150 provides at least 80% power for detecting a population correlation ≥0.23 (α=0.050 two-sided test). Modeling will be matched to the conditional distribution of the response. Where homogeneous Gaussian errors are indicated, standard regression models will be used. Where data exhibit a multi-fold range, log transformations will be assessed for usefulness. In a sample of 106 human volunteers (Thomson *et al.* 2007), the FFQ estimated that intake ranged >50-fold (5.7-390 g/day). Proportional odds models (or other cumulative link models) will be used for the ordinal response, RNR protein expression. Generalized linear models (with allowance for observed over-dispersion) may be used for data exhibiting appropriate mean-variance relationships and/or non-linearities in mean response. Other variables of interest from 24-h dietary recalls will be treated in a similar way.

Anticipated results, potential pitfalls, alternative strategies Based on the central hypothesis and preliminary data, it is anticipated that mechanistic findings in aim 1 and preclinical outcomes from aim 2 will be corroborated in human subjects with respect to RNR expression changes (R2>p53R2>>R1) at high versus low intake of green leafy vegetables. It is further anticipated that LC-MS/MS data will help to validate the intake levels of chlorophylls, or the associated metabolites and breakdown products. This is a small proof-of-concept trial that does not seek to inform on *clinical outcome*; thus, we are not testing the hypothesis that spinach or other leafy vegetables lower RNR expression and therefore reduce the incidence of colon polyps. However, changes in RNR expression with respect to intake of green leafy vegetables could inform and provide the basis for an intervention with isolated chlorophyll, as in Jubert *et al.* (2009), prior to a larger study powered for clinical outcomes such as polyp inhibition.

Based on data from aims 1 and 2, prioritization will be made among molecular endpoints, starting with R2 and p53R2. Individuals with low/no Chl intake would be expected to have high RNR expression levels in tumors versus normal colon biopsies, as evidenced in immunoblot/immunohistochemistry studies. High Chl intake will result in loss of R2 and/or p52R2 levels, affecting both adenocarcinoma and invasive stages (Liu *et al.* 2007), with a possible shift to early adenomas. By 'piggy-backing' on the ongoing clinical trial, information will be captured on epigenetic endpoints, such as histone marks (Fig 9). These findings would be integrated with data from aims 1 and 2 on specific histone marks altered by Chl or Ce6 on the promoter regions of *RRM2* or *RRM2B*, and corresponding changes in mRNA transcripts. Parallel studies would examine proliferation and apoptosis markers in polyps and normal colon biopsies in subjects reporting high *versus* low intake of Chl-rich foods.

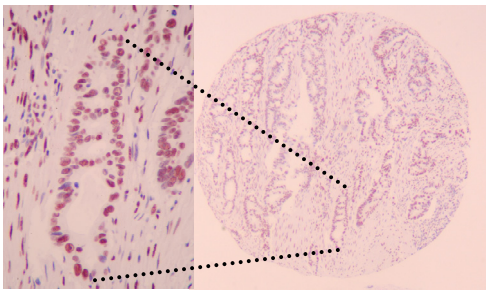

Fig 9 Immunostaining of histone H3K9 acetylation in human colon cancer tissue microarrays. C.V. Löhr, unpublished data.

In the context of the central hypothesis, individuals reporting high intake of green leafy vegetables would be expected to have greater levels of chlorophylls or their metabolites/breakdown products in plasma and tissue samples than people reporting little or no consumption of such foods. Despite using

a validated FFQ and 24-h recalls, over- and under-reporting of green leafy vegetables is possible. Even with state-of-the art analytical approaches (Fig 10), LC-MS/MS methodologies optimized in mice (under aim 2) might be less suitable for biopsies and plasma samples from human subjects. This could be due to inconsistencies in collecting and storing tissue samples for assessment of chlorophylls (which are notoriously sensitive to light and oxygen), or to novel metabolites in humans that were not identified in preclinical models. To begin to address these questions, LC-MS/MS studies will be undertaken with human tissue samples spiked with isolated chlorophylls, and their fate examined under storage conditions used in the clinical trial. Key comparisons will be between colon polyps and normal-looking colon biopsies. PBMCs will inform on RNR status in normal cells in the circulation, and any differences with *normal colon* biopsies might reflect systemic *versus* GI distribution of the active compounds. Given that preliminary data showed RNR changes in cancer cells but not in normal cells following treatment with the test agents, it will be important to ascertain whether RNR levels in PBMCs inform on RNR expression in polyps, thereby helping to define the most relevant and robust clinical biomarkers.

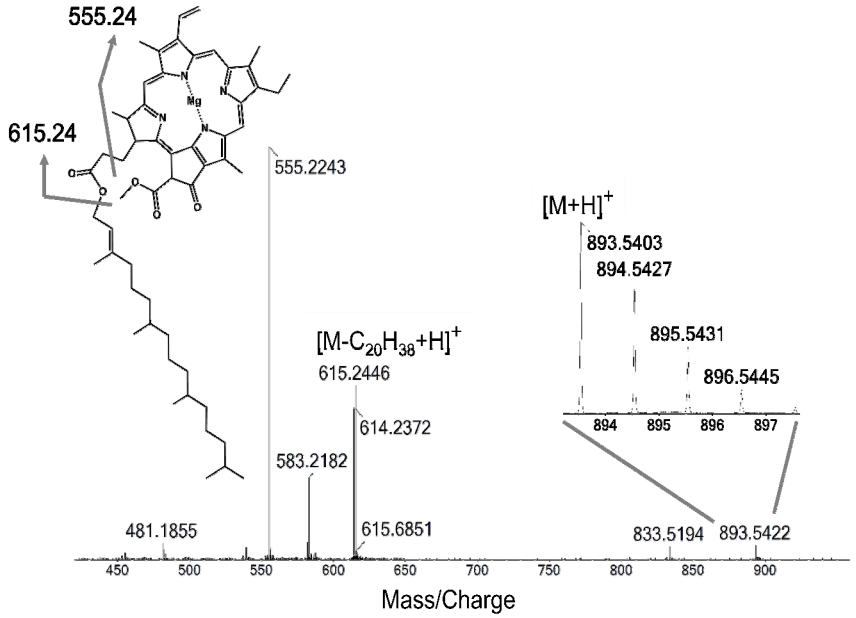

Fig 10 LC-MS/MS of Chl *a* from broccoli. Insert shows the protonated molecular ion. The fragment ion at *m/z* 615.24 indicates loss of the phytyl chain from Chl *a*. UPLC-MS/MS on a 5600 AB Sciex Triple TOF instrument, following the method of Fu *et al.* 2012. Spectra were acquired in the Information Dependent Acquisition mode and analyzed using PeakView software. S. Wickramsekara and C. Maier, unpublished preliminary data.

Tentative schedule for completing the Specific Aims

Aim	Year 1	Year 2	Year 3	Year 4	Year 5
aim 1a	EPR studies of Chl, chlorins with R1, R2, p53R2 - - - - - - - - - - - - - - - - -				
aim 1b	*In silico* modeling of Chl, chlorins with R1, R2, p53R2 - - - - - - - - - - - - - - - -				
aim 1c		Enzyme kinetics studies with Chl, Ce6- - - - plus other chlorins from aim 1b- - - -			
aim 1d	ChIP assays of *R2* and *p53R2* promoters after Chl, Ce6 - - - - - - plus other chlorins from aim 1b - - -				
aim 1e		mRNA stability and turnover mechanisms (role of miRNAs) - - test other chlorins from aim 1b - - - - - - - -			
aim 1f		Normal *versus* cancer cells (colon, lung), siRNA and overexpression, S-phase arrest, apoptosis, etc - - - - - -			
aim 2a		R2 and p53R2 mice/lung tumor suppression - - - - -			
aim 2b			R2 or p53R2 mice±DMH; Chl/Ce6 dose-response - - -		
aim 2c			RNR changes (etc) in mouse tissues from aims 2a and 2b - - - - - - - - - - - -		
aim 2d			dNTP pool imbalances (lung, colon, other tissues) of mice from aims 2a,b		
aim 2e			O^{18}-labeled Chl, Ce6 in mouse urine, plasma, tissue (LC-MS/MS analyses)		
aim 3a	R1,R2, p53R2 levels in human polyps, normal colon, PBMCs (qRT-PCR, IHC, Western blots) - - - -				
aim 3b	R1, R2, p53R2 levels in human PBMCs -				
aim 3c				Chl metabolites and breakdown products in plasma, tissue biopsies (LC-MS/MS analyses)- - - - -	