Aerosolised 5-azacytidine suppresses tumour growth and reprogrammes the epigenome in an orthotopic lung cancer model

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Background: Epigenetic silencing by promoter methylation and chromatin remodelling affects hundreds of genes and is a causal event for lung cancer. Treatment of patients with low doses of the demethylating agent 5-azacytidine in combination with the histone deacetylase inhibitor entinostat has yielded clinical responses. The subcutaneous dosing route for consecutive days and reduced bioavailability of 5-azacytidine because of inactivation by cytidine deaminase may limit the expansion of epigenetic therapy into Phase III trials. To mitigate these barriers, an aerosol of 5-azacytidine was generated and characterised.

Methods: The effect of aerosol vs systemic delivery of 5-azacytidine on tumour burden and molecular response of engrafted lung tumours in the nude rat was compared.

Results: Pharmacokinetics revealed major improvement in the half-life of 5-azacytidine in lung tissue with aerosol delivery. Aerosolised 5-azacytidine significantly reduced lung tumour burden and induced global demethylation of the epigenome at one-third of the comparable effective systemic dose. High commonality for demethylation of genes was seen in tumours sampled throughout lung lobes and across treated animals receiving the aerosolised drug.

Conclusion: Collectively, these findings show that aerosolised 5-azacytidine targets the lung, effectively reprogrammes the epigenome of tumours, and is a promising approach to combine with other drugs for treating lung cancer.

Epigenetic-mediated silencing of genes by cytosine DNA methylation in CpG islands localised in promoter regions and chromatin remodelling involving the recruitment of transcriptional corepressors and modification of histone tails is a causal event for the initiation and progression of lung cancer (Jones and Baylin, 2002; Herman and Baylin, 2003). Silencing of hundreds of genes affecting all aspects of cell regulation has been detected through candidate and whole-genome approaches (Belinsky, 2005; Brenna et al, 2007; Tessema et al, 2009). The minimal efficacy of conventional chemotherapy for advanced stage lung cancer and the reversibility of epigenetic-mediated gene silencing by pharmacologic agents has stimulated clinical efforts to evaluate the efficacy of epigenetic therapy. Cytosine methylation appears dominant in transcriptional repression, and inhibitors of the cytosine DNA methyltransferases, vidaza (5-azacytidine (5-Aza)) and decitabine (5-aza-2'-deoxycytidine) can induce in vitro re-expression of genes silenced through promoter hypermethylation (Jones and Baylin, 2002; Herman and Baylin, 2003). Importantly, although inhibitors of histone deacetylation (HDAC) are not very effective in inducing re-expression of genes silenced by promoter hypermethylation, such inhibitors can synergise with demethylating agents to relieve transcriptional repression (Cameron et al, 1999). The initial use of demethylating agents suggested that they were too toxic and not efficacious as cancer cytotoxic agents. However, laboratory findings revealed that the concentration of these drugs needed to induce gene demethylation and re-expression was much lower than that required to produce maximal cytotoxicity (Cameron et al, 1999). In fact, cell cycle arrest in response to cytotoxicity will reduce gene
demethylation owing to the requirement of incorporation of these cytidine analogs into replicating DNA. Thus, these drugs were reassessed at doses much lower than the maximum-tolerated dose and proved to be a potent therapy for myelodysplasia (MDS), a precursor state to acute myelogenous leukaemia, leading to their approval by the FDA for treatment of these diseases (Silverman et al., 2002; Yang et al., 2006). Clinical trials with DNA-demethylating agents combined with HDAC inhibitors are also showing promising responses in the treatment of myeloid malignancies (Gore et al., 2006).

This low-dose strategy was extended in a Phase I/II trial in which patients with refractory advanced non-small-cell lung cancer were treated with 5-Aza and the HDAC inhibitor entinostat. For the Phase II component, patients received 40 mg m⁻² per day 5-Aza subcutaneously on days 1 to 6 and 8 to 10 with entinostat taken orally on days 3 and 10 of each 28-day cycle. This therapy was well tolerated, and importantly, objective (complete or partial) responses were observed in two patients. Furthermore, 4 of 19 patients had major objective responses to subsequent anticancer therapies given immediately after epigenetic therapy. Although pretreatment tumour tissue was not available, the methylation state of a four-gene panel previously associated with rapid tumour recurrence was studied in serial plasma specimens (Juergens et al., 2011). Demethylation of these genes was associated with improved progression-free survival.

The expansion of epigenetic therapy into Phase III trials or to adjuvant therapy may be constrained by the required continuous daily subcutaneous dosing schedule that is also associated with some low-grade toxicity at the injection site (Juergens et al., 2011). Oral formulations are problematic because of hydrolysis and catabolism by cytidine deaminase; however, a recent film-coated formulation of 5-Aza has shown some bioavailability and response in a Phase I trial of MDS (Garcia-Manero et al., 2011). Cytidine deaminase expression is also highest in the liver where conversion of 5-Aza to its uridine counterpart occurs with subcutaneous administration as well and this reduces the concentration of active drug seen by the lung (Ho and Frei, 1971). Furthermore, sequence variants within the promoter region of cytidine deaminase have been associated with a threefold variation in enzymatic activity (Fitzgerald et al., 2006). Finally, increased activity of cytidine deaminase was associated with reduced half-life of 5-Aza and worse outcome for male patients with MDS (Mahfouz et al., 2013). These barriers to expanding the use and improving the response to epigenetic therapy could be mitigated by aerosolised delivery that deposits drugs directly to the airways of the lungs by gravitational sedimentation and inertial impaction and to the peripheral region by diffusion. The absorption of the aerosol into the pulmonary vasculature also avoids hepatic first-pass metabolism. Thus, individual differences in cytidine deaminase activity would be eliminated and lower doses of drug could be delivered directly to the lungs to achieve equivalent clinical effects.

The development and testing of different exposure routes for therapy requires an animal model that best mimics human lung cancer and barriers for treatment. We have developed an orthotopic lung cancer model in which xenografts of human lung cancer-derived cell lines are efficiently engrafted throughout the lungs of the Rowett nude rat (March et al., 2001; Belinsky et al., 2011). Our first studies demonstrated that combination therapy with systemic delivery of 5-Aza and entinostat at doses and schedule similar to the Phase II clinical trial were synergistic in suppressing tumour growth and induced reprogramming of the epigenome as detected by gene demethylation and re-expression. The current study extends this work by generating and characterising an aerosol of 5-Aza and then comparing the effect of aerosol vs systemic delivery of 5-Aza on tumour burden and molecular response of engrafted lung tumours in the nude rat.

### MATERIALS AND METHODS

#### Treatment protocols and tumour cell implantation.

Male Rowett nude rats (Crl:NIH-ru), 8–10 weeks old were obtained from Frederick Cancer Research and Development (Frederick, MD, USA). For pharmacokinetic studies, rats (three per group per time point) were treated with a single dose of 5-Aza (Sigma, St Louis, MO, USA) dissolved in saline administered systemically by intraperitoneal injection (2 mg kg⁻¹) or as an aerosol (0.6 mg kg⁻¹ inhaled deposited dose) for 90 min via a nose-only nebuliser. The aerosol dose delivered to the rat lungs was calculated based on the aerosol concentration times the respiratory minute volume times the exposure period times the deposition fraction divided by body weight (Bide et al., 2000). Animals were serially killed for collection of plasma and lung tissue at 11 time points over 96 h after exposure. Calu-6 cells (containing K-ras and p53 mutations) obtained from American Type Culture Collection (Manassas, VA, USA) were cultured and instilled via orotracheal intubation as described (March et al., 2001; Belinsky et al., 2011). Three weeks following instillation of tumour cells, rats (n = 20 per group) were treated with vehicle (saline), 5-Aza by intraperitoneal injection (2 mg kg⁻¹), or aerosol (0.6 mg kg⁻¹) for 4 consecutive days over 4 consecutive weeks as described (Belinsky et al., 2011). An additional six rats that did not receive tumour cells were included to determine tumour-free lung weights for comparison with treatment groups.

#### Inhalation exposure, aerosol characterisation, and pharmacokinetics.

All animal procedures were conducted under protocols approved by the Institutional Animal Care and Use Committee at Lovelace Respiratory Research Institute, which is accredited by the Association for Assessments and Accreditation of Laboratory Animal Care International. Rodents were housed in nose-only tubes during the exposures. Chamber oxygen content was collected directly from an enclosed nose-only exposure port. Temperature readings were obtained from a monitor placed within the secondary containment that enclosed the exposure chamber. All flow rates and system pressures (e.g., pressure in the sample, exhaust, and lines) were monitored by a rotameter (flow) and magnetohelic (pressure) enclosure in a control panel. Flow rates were point-calibrated (calibrated at a specific flow rate) with a flow meter. The aerosol of the solution nebuliser formulation (5 mg ml⁻¹) was generated with three Pari LC Plus nebulisers (Pari Respiratory Equipment, Miliothian, VA, USA). The Pari LC Plus nebulisers were operated with clean, regulated inlet air at a pressure of 20 psi, which resulted in an output of ∼151 min⁻¹. The 5-Aza aerosol was directed through ∼24 in. of a 1.58-cm diameter stainless-steel tube into a 52 port flow-past exposure chamber. The chamber exhaust was maintained at ∼141 min⁻¹.

Exposure monitoring was conducted by collecting material onto Pallflex 47-mm membrane filters (Pall Corporation, Port Washington, NY, USA). After sample collection, the filters were analysed gravimetrically by differential weights. Particle size (mean mass aerodynamic diameter (MMAD)) of the nebulised formulation was measured using a Mercer-style impactor (In-Tox Products Inc., Moriarty, NM, USA) operated at approximately 2.01 min⁻¹. The impactor sample was collected directly from the breathing zone of the nose-only exposure system. The analysis of impactor data and the calculation of MMAD were performed as described by Thiel (2002).

Plasma and lung samples were prepared via a solid-phase extraction before LC/MS/MS analysis. Chromatographic separation was conducted with an Agilent 1200 HPLC fitted with a C₁₈ column held at 25 °C. The shallow gradient used buffered water and methanol to elute 5-Aza in ∼3 min (Kissinger and Stemm, 1986). Detection was performed with MS/MS analysis using an electrospray ionisation source in the positive mode.
Tissue collection and estimation of tumour burden. Before being killed, four animals from each treatment group and vehicle were randomly selected for collection of tumours (n = 5 per animal) for molecular assays. After weighing the remaining lungs, they were inflated with 10% neutral-buffered formalin at a constant hydrostatic pressure of 25 cm for 6 h. Paraffin-embedded lungs were sectioned at 5 μm thickness and stained with haematoxylin and eosin. Our previous study demonstrated that treatment-related reduction in tumour burden was highly correlated with estimates of tumour volume (Belinsky et al., 2011). Therefore, tumour burden was used to assess the response to systemic vs aerosol delivery of 5-Aza. Tumour burden was quantitated 7 weeks following instillation of Calu-6 cells as the change in normal lung weight compared with tumour-bearing lung weights in the vehicle and 5-Aza treatment groups.

Gene expression. RNA was isolated from lung tumours (five per animal) collected from four rats per treatment group and vehicle following TRI-reagent (Sigma, St Louis, MO, USA) instructions. Total RNA (1 μg) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer’s protocol. RT-qPCR was performed with the ABI PRISM 7900HT and inventoried Taqman assays (Applied Biosystems). Experiments were normalised to GAPDH or β-actin. Results were repeated in three separate experiments. Data were analysed with respect to a calibrator sample using the 2−ΔΔCt method and reported as relative quantity.

Gene methylation profiling. Bisulphite-modified DNA isolated from vehicle, 5-Aza intraperitoneally, or 5-Aza aerosol-treated Calu-6 tumours (four animals per group with three tumours per animal) were hybridised to the Illumina Methylation450 (HM450K) Beadchip (Illumina, San Diego, CA, USA). Bisulphite-modified DNA from normal bronchial epithelial cell lines obtained from three, cancer-free never smokers was included to identify genes methylated in normal cells.

Statistical analyses. Pharmacokinetic analysis was performed with WinNonLin (Pharsight Corp., Cary, NC, USA; Version 5.0.1) using non-compartmental analysis of plasma and lung samples that were above the limit of quantitation for the analytical assay. This analysis averages the amount of 5-Aza from the three animals at each time point to define the area under the curve, half-life, and concentration of the drug for systemic and aerosol delivery. Thus, no error values are given with the values presented in Table 1. The two-sample t-test and analysis of variance were used to compare the two treatment groups and the two groups with the vehicle, respectively. To check the appropriateness of the statistical methods, analyses were re-run with transformed data if appropriate or using the Kruskal–Wallis test.

RESULTS

Characterisation of the 5-Aza aerosol and pharmacokinetics. Because aerosol delivery should be more potent per unit dose, our target dose for 5-Aza was 75% less than the effective systemic dose of 2 mg kg−1. The actual calculated delivered dose of 5-Aza was 0.6 mg kg−1 based on an average aerosol concentration of 0.1 mg l−1 delivered over 90 min and pulmonary deposition assumed to be approximately 10% (Kuehl et al., 2012). Particle size analysis revealed an average MMAD of 0.71 μm with a geometric standard deviation of 1.96, values optimal for achieving good pulmonary distribution to the airways and parenchyma. The pharmacokinetic profile was determined in the blood and lung tissue following a single dose of 5-Aza delivered systemically (2 mg kg−1) vs via the nebulised aerosol (0.6 mg kg−1). As expected, the area under the curve for overall drug retention in blood was significantly greater for systemic than aerosol delivery, whereas the opposite was seen for delivery to the lungs (Figure 1 and Table 1). However, the half-life for 5-Aza in blood and lung tissue was increased 2.9- and 13-fold by aerosol delivery compared with systemic exposure (Table 1).

Aerosol delivery of 5-Aza reduces tumour burden and markedly increases the expression of the proapoptotic gene Bik. The delivery of 5-Aza systemically or via aerosol over 4 weeks resulted in an equivalent (34% vs 29%), significant reduction in tumour burden (Table 2). Rats also showed similar weight gain of 15–20%, irrespective of treatment route. Previous histological characterisation of Calu-6 tumours in the nude rat revealed two morphologically distinct cell populations and this was recapitulated in the current study (Belinsky et al., 2011). One population was composed of well-differentiated mucus-secreting adenocarcinoma cells and rare mitotic figures. A second interspersed cell population was composed of solid cords and nests of pleomorphic cells that occupied up to 75% of the tumour mass. These cells displayed high mitotic activity with up to 10 mitotic figures per ×40 field. Previously, the reduction in tumour burden seen with systemic delivery of 5-Aza was largely mediated through ablation of the pleomorphic cell population (<5% of the residual tumour) with a portion of the well-differentiated tumours remaining (Belinsky et al., 2011). Inhaled delivery of 5-Aza was equally effective as systemic delivery in removing the pleomorphic cell population (not shown). Expression of the proapoptotic genes studied previously (Belinsky et al., 2011), Bad, Bak, and Bok, were increased approximately threefold, irrespective of the exposure route in tumours when normalised to GAPDH or β-actin (Figure 2; not shown). Aerosol delivery of 5-Aza caused a more profound effect.

Table 1. Pharmacokinetic parameters for aerosol and systemic delivery of 5-Aza

| Delivery route | Plasma AUC (h × ng ml−1) | Half-life (h) | Concentrationmax (ng ml−1) |
|---------------|---------------------------|--------------|---------------------------|
|               | Plasma Lung | Plasma Lung | Plasma Lung |
| Systemic      | 19.128 1082 | 0.42 0.14 | 15 2149 893 7883 |
| Aerosol       | 1887 8460 | 1.24 1.84 | 7 2147 7883 |

Abbreviations: 5-Aza = 5-azacytidine; AUC = area under the curve; Max = maximum.
on the expression of Bik, a major proapoptotic gene in lung epithelial cells (Mebratu et al., 2008) than systemic delivery (Figure 2). The cyclin-dependent kinase inhibitor p21 (WAF1) functions as a regulator of cell cycle progression and senescence and its expression was also increased to a similar extent following systemic or aerosol delivery of 5-Aza (Figure 2).

**Treatment with 5-Aza induces global demethylation of the epigenome.** The HM450K array was used to compare the epigenetic therapy delivered by aerosol reduces lung tumour burden. Values are the mean ± s.e.m. from 20 tumours per group (four rats per group). All individual assays were conducted in triplicate. *P<0.001 compared with systemic delivery.

| Treatment group | N | Delivery route | Lung weight (g), mean ± s.d. | Adjustment for control, weight (± s.d.) |
|-----------------|---|----------------|-------------------------------|---------------------------------------|
| Control b       | 6 | NA             | 1.74 ± 0.21                   | NA                                    |
| Vehicle         | 16| NA             | 4.52 ± 1.82                   | 2.78 ± 1.57                           |
| 5-Aza           | 18| Systemic       | 3.58 ± 0.95                   | 1.84 ± 0.82*                          |
| 5-Aza           | 17| Aerosol        | 3.72 ± 0.82                   | 1.98 ± 0.77*                          |

Abbreviations: 5-Aza = 5-azacytidine, NA, not applicable. *P<0.05 compared with vehicle. bSize of treatment groups differ because of engraftment. Instillation through the orotracheal tube sometimes misses the trachea, resulting in placement into the oesophagus. bControl animals received no tumour cells.

Genes were seen with systemic treatment in all tumours. Relaxing the criteria to commonly demethylated in 75% of tumours identified 501 and 275 genes commonly associated with aerosol vs systemic exposure. Demethylation of all 275 genes following systemic exposure was also seen in tumours treated by aerosol delivery. Genes showing demethylation were involved in key cell functions (see Supplementary Table S1 for a complete list) that included adhesion and invasion (e.g., cadherins, laminins, keratins), migration (e.g., SLIT family), apoptosis (e.g., Elmo3), and cell trafficking (e.g., CXCL chemokines). Moreover, genes encoding for transcription factors (e.g., FOX, HOX, GATA families) were demethylated. Five genes of diverse function and exhibiting average demethylation of ≥40% were selected to assess quantitatively the resulting change in expression compared with vehicle-treated tumours. Expression increases of 2- to 3.3-fold were seen when normalised to GAPDH or β-actin (Figure 4; not shown).

**Figure 2. Delivery of 5-Aza by aerosol or systemically increases the expression of proapoptotic genes and p21.** TaqMan PCR was used to quantify the change in expression of the proapoptotic genes Bad, Bak, Bok, and Bik and p21 in tumours from animals treated with 5-Aza delivered as an aerosol or systemically. The fold increase in expression is relative to expression seen in vehicle tumours (set to 1) and normalised to glyceraldehyde dehydrogenase (GAPDH). Values are the mean ± s.e.m. from 20 tumours per group (four rats per group).

**DISCUSSION**

These studies show that 5-Aza delivered as an aerosol can effectively reduce lung tumour burden and induce global demethylation of the epigenome at more than one-third of the
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The improved pharmacokinetic profile seen in the tumours collected throughout the lung lobes of rats receiving the aerosolised 5-Aza. Our studies did not address survival as an outcome because the aim was to focus first on improving the pharmacokinetic profile for this drug. However, Mahesh et al (2010) has shown that intratracheal delivery of 5-Aza at equivalent doses increased the survival of mice bearing orthotopic H460 and H358 xenografts. Our future studies will include an HDAC inhibitor such as entinostat, as this combination is being used clinically and has shown superior efficacy to a demethylating agent alone in our murine and rat models (Belinsky et al, 2003, 2011).

On average, approximately 50% of the genes classified by the HM450K array as methylated in the Calu-6 tumours responded to the aerosol delivery. These genes as exemplified by our re-expression studies participate in many of the critical cell regulatory processes. CXCL5, a chemokine important in regulating cell migration and tumour metastasis, was demethylated and re-expressed following aerosolised 5-Aza. This chemokine along with two other family members, CXCL12 and CXCL14 (also demethylated following therapy), are silenced in more than 85% of primary lung adenocarcinomas (Tessema et al, 2010). Moreover, our previous work showed that forced expression of CXCL14 increased the expression of cell cycle inhibitor and proapoptosis genes and markedly abrogated growth of tumour xenografts (Tessema et al, 2010). Oligodendrocyte transcription factor 1 (OLIG1), whose silencing is significantly associated with poor prognosis for lung cancer patients, is demethylated by >70% and re-expressed with treatment (Brena et al, 2007). Finally, protocadherin-20, a homophilic cell adhesion member of the protocadherin family that is important in calcium-dependent cell–cell adhesion and signal transduction and silenced in 50% of tumours, was re-expressed by epigenetic therapy (Tang et al, 2012). These examples highlight the robust response seen with aerosol delivery of 5-Aza with regard to reactivation of key tumour suppressor genes to impact the growth of the Calu-6 line that also harbours mutations in the K-ras and p53 genes.

The promise of aerosol delivery for treatment of lung cancer is also substantiated through Phase 1 clinical trials with cisplatin and gemcitabine, two drugs used as first-line therapy for non-small-cell lung cancer. Cisplatin was encapsulated in lipid vesicles that are dispersed throughout the aqueous phase and delivered via nebuliser to patients with advanced and/or metastatic lung cancer who had progressed after multiple other therapies (Wittgen et al, 2007). Pharmacokinetic results showed very low plasma levels of cisplatin and no nephrotoxicity that is associated with intravenous delivery, whereas dose-limiting toxicity of the aerosolised cisplatin was not reached. Importantly, 12 of 16 patients had stable disease for up to eight, 21-day cycles of treatment, whereas the remaining patients progressed on therapy. Gemcitabine was reconstituted in saline and delivered by nebuliser in a dose escalation study to lung cancer patients 1 day per week for 9 consecutive weeks (Lemarie et al, 2011). The initial dose of drug included 99mTc-diethylene triaminpentaoctic acid to facilitate pharmacokinetic assessment of delivery to the lungs. On average, the total dose of gemcitabine delivered to the lungs was 42 ±16%, low plasma levels were observed, and no haematologic toxicity, nephrotoxicity, or neurotoxicity were observed. A minor response was seen in one patient, stable disease in four patients, and progressive disease in four patients. These studies demonstrate the feasibility and safety of aerosolised delivery of chemotherapeutics and support additional assessment in patients with less severe and extensive disease.

The rapid improvement in aerosol formulation and delivery devices could greatly change the landscape for therapy against lung cancer (Nanjwade et al, 2001; Gagnadoux et al, 2008). Clearly, the delivery of cytotoxins, drugs, and small molecules targeting genes or pathways to the primary tumour with minimal systemic toxicity will allow the testing of new combinatorial approaches and more
intensive dose schedules. Dry powder inhalers offer improved stability and bioavailability for some drugs and the opportunity to combine drugs into a single formulation. For example, nanoparticles of paclitaxel have been assembled to form low-density microparticles in the presence of cisplatin in solution, a process that actually accelerated the dissolution of the paclitaxel (El-Gendy and Berkland, 2009). Delivering drugs in microparticles and nanoparticles can also overcome solubility constraints, facilitate sustained release, and achieve relatively uniform distribution of drug dose within the lung parenchyma for peripheral tumours (Willis et al, 2012; Meenach et al, 2013). Combination therapy with systemically delivered 5-Aza and the histone deacetylase inhibitor entinostat were synergistic for reducing tumour burden and further effecting global gene expression of Calu-6, A549 (K-ras mutant), and H1975 (EGFR mutant) tumours in our orthotopic lung cancer model (Belinsky et al, 2011). Moreover, hundreds of genes were demethylated highlighted by the re-expression of polycomb-regulated genes coding for transcription factor binding proteins. In addition, highly significant gene expression changes were seen in key regulatory pathways involved in cell cycle, DNA damage, apoptosis, and tissue remodelling.

Similar studies combining entinostat with aerosolised delivery of 5-Aza can now be pursued to see if the response to this drug combination is magnified further, given the improved pharmacokinetic profile for this drug with respect to lung half-life and commonality for demethylation between tumours. The target population for translating this work into the clinic is likely those patients with cancer largely confined to the lungs (Stages II and III), whereas aerosol delivery of 5-Aza could also benefit Stage I patients after resection as an adjuvant therapy to prevent a second primary cancer. Our studies cannot address another important question, whether the systemic exposure following aerosol delivery, albeit at a lower doses than subcutaneous delivery, but avoiding first-pass metabolism through the liver, will effect occult metastases likely present in Stage III lung cancer patients. This question will necessitate clinical trials in this patient population. Finally, it is attractive to propose chemoprevention targeting the epigenome for smokers identified to be at high risk for lung cancer through the detection of methylated genes in their sputum (Belinsky et al, 2006; Leng et al, 2012). Although 5-Aza cannot be used in cancer prevention because of its mutagenic properties, the development of aerosols for dietary polyphenols (whose inherent low bioavailability may limit effectiveness) that show in vitro activity toward inhibiting cytokine DNA methyltransferases may be a promising alternative (Fang et al, 2007).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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