POLYAMINES AND CANCER: OLD MOLECULES, NEW UNDERSTANDING

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Abstract | The amino-acid-derived polyamines have long been associated with cell growth and cancer, and specific oncogenes and tumour-suppressor genes regulate polyamine metabolism. Inhibition of polyamine synthesis has proven to be generally ineffective as an anticancer strategy in clinical trials, but it is a potent cancer chemoprevention strategy in preclinical studies. Clinical trials, with well-defined goals, are now underway to evaluate the chemopreventive efficacy of inhibitors of polyamine synthesis in a range of tissues.

UREA CYCLE
The key metabolic pathway in mammals for eliminating cellular breakdown products containing nitrogen.

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Polyamines are organic cations that are derived from amino acids and are found in all organisms. Polyamine research dates back several centuries (TIMELINE 1). In 1678, Van Leewenheuk identified crystals in semen that were later identified as the tetraamine spermine; the diamine putrescine was first identified in microbes in the late 1800s, and the triamine spermidine was identified in the early twentieth century[1]. Putrescine, spermidine and spermine are the main polyamines found in prokaryotes and eukaryotes, but other amines have been identified (for example, in extreme thermophiles)[1].

In some bacteria, putrescine derives from arginine decarboxylation through a two-step mechanism[1]. In mammals, the diamine putrescine is synthesized in the cytoplasm as a consequence of the decarboxylation of ornithine, an amino acid that is not found in proteins and that is produced as part of the UREA CYCLE, which involves both cytosolic and mitochondrial enzymes (FIG. 1). Putrescine is the precursor used in spermidine and spermine biosynthesis. All urea-cycle enzymes are expressed in the liver and, to a lesser degree, in the intestinal mucosa[2]. In addition, certain urea-cycle enzymes, including those involved in polyamine metabolism, are widely expressed in all tissues. So, although urea-cycle enzymes are expressed primarily in the liver and intestine, polyamines are made in all tissues. Polyamines are also obtained from the diet (foods that are high in polyamines include cheese and red meat[3]) and from other sources, such as intestinal bacteria[1,4]. These externally derived polyamines are transported into cells from extracellular spaces.

Genetic studies in the 1980s by Herbert and Celia Tabor and colleagues demonstrated that polyamines are essential for optimal growth and viability in bacteria[5] and yeast[6]. John Cleveland and colleagues have recently extended this paradigm to mammals, with the observation that the gene that encodes ornithine decarboxylase (ODC) — the enzyme required for the first stage in polyamine synthesis (FIG. 1) — is essential in mice. The mechanism of this requirement seems to involve the suppression of apoptosis by ODC in the developing mouse embryo[7]. Polyamines have been widely implicated in the growth and development of a range of mammalian tissues and in remodelling processes associated with tissue repair (BOX 1). Interestingly, polyamine synthesis is downregulated as cells become senescent[8] in many tissues in adults[9].

The association of increased polyamine synthesis with cell growth and cancer was first reported in the late 1960s. Russell and Snyder reported high levels of ODC activity in regenerating rat liver and in several human cancers[10]. Subsequently, Anderson and Heby observed similar changes in tumour-cell polyamine levels[11]. Russell and others went on to show that polyamine levels were increased in the urine of patients with cancer, although studies so far have not proven that urine polyamine levels

Summary

- Polyamines are naturally occurring organic cations found in plants, animals and microbes. They are formed by the enzymatic decarboxylation of the amino acids ornithine or arginine.
- Ornithine decarboxylase (ODC) is the first enzyme in the polyamine synthesis pathway in mammals and is the target for difluoromethylornithine (DFMO), a substrate analogue and specific inhibitor that irreversibly inactivates ODC when it binds to the active site of the enzyme.
- ODC and several other polyamine metabolic proteins are essential for normal cell and tissue functions, including growth, development and tissue repair. ODC and polyamine content are increased in many cancers arising from epithelial tissues, such as the skin and colon.
- Polyamines exert their effects in eukaryotic cells in part by regulating specific gene expression.
- In murine and human colonic mucosal tissue, ODC is negatively regulated by the adenomatous polyposis coli (*APC*) tumour-suppressor gene. *APC* is mutated or deleted in the germline of people with familial adenomatous polyposis (FAP), a genetic syndrome associated with a high risk of colon cancer. *APC* is also mutated or deleted in somatic colon epithelial cells in most sporadic, or non-genetic, forms of colon cancer.
- Loss of APC function causes an increase in ODC activity and polyamine biosynthesis, and tumour formation in $Apc^{Min/+}$ mice, a murine model of human FAP. Treatment of $Apc^{Min/+}$ mice with DFMO suppresses intestinal tumour formation.
- Several non-steroidal anti-inflammatory drugs (NSAIDs), the use of which is associated with decreased risk of epithelial cancers, activate the transcription of spermidine/spermine N^1-acetyltransferase, the first enzyme in the polyamine catabolic pathway. Experimental studies indicate that combinations of DFMO and NSAIDs are potent inhibitors of colon and intestinal cancer development in murine models.
- Clinical studies have shown that DFMO is well tolerated and can prevent the development of precancerous lesions in the skin. Several large randomized trials involving the skin, colon and other organ sites are underway.

are an adequate prognostic marker of cancer[12]. The role of urinary polyamines as a predictive marker for response to therapy remains to be established.

Early work in the lab of Roswell Boutwell showed that chemical promoters of skin carcinogenesis induced ODC[13], and ODC activity is now known to be induced in normal tissues by a range of environmental and genetic cancer risk factors. For example, ultraviolet light induces *Odc* gene expression in rodent models of squamous-cell skin cancer, and inhibitors of ODC suppress this process[14]. In addition, asbestos, the causative agent of the deadly lung cancer mesothelioma, is a potent inducer of ODC[15]. ODC is also regulated by androgens in the prostate gland[16], and the gene encoding ODC is markedly induced in human prostate cancer[17]. In addition to these links between ODC activity and cancer risk factors, the use of pharmacological inhibitors of polyamine metabolism has implicated polyamines in a range of cancers.

Difluoromethylornithine (DFMO), which irreversibly inactivates ODC, is the most widely studied example of a polyamine-metabolism inhibitor that suppresses cancer development in animal models[18] and has been evaluated in clinical trials. Agents that target other polyamine metabolic enzymes (FIG. 1) are also potent inhibitors of cancer growth in experimental model systems[19,20], and some of these agents are either under evaluation or have been evaluated in human clinical cancer therapeutic trials[21,22].

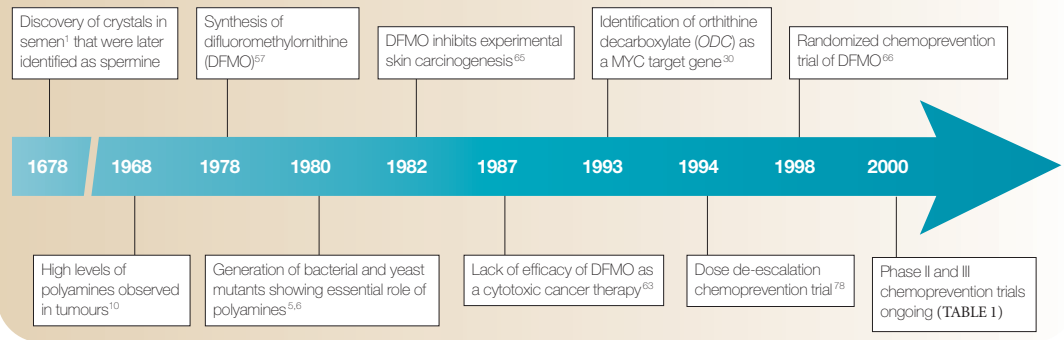
Despite the fact that the link between polyamines and cancer has been known for more than 30 years, until recently, knowledge of the specific mechanisms by which polyamine metabolism is altered during carcinogenesis has been lacking, as has evidence to indicate that polyamines have a causative rather than associative role in cancer. New advances in understanding the roles of polyamines in cancer are discussed here, as are the uses of inhibitors of polyamine metabolism for cancer treatment and prevention.

Mechanisms of polyamine upregulation in cancer

The mechanistic basis of the association between cancer risk factors and polyamine metabolism has become clearer during the past decade, particularly through studies of colon cancer. The relationships between two tumour-suppressor genes and two oncogenes implicated in colon carcinogenesis and genes regulating polyamine levels in the colonic mucosa are shown in FIG. 2.

ODC is upregulated in the intestinal mucosa of individuals with familial adenomatous polyposis (FAP)[23], a heritable form of colon cancer. The tumour suppressor adenomatous polyposis coli (*APC*) is mutated or lost in the germline of individuals with FAP[24,25] and in somatic intestinal epithelial cells that develop into colonic polyps in individuals who are not genetically predisposed to colon cancer[26]. In colon tumour cells, loss of APC function leads to increased expression of the *MYC*[27] oncogene, aberrant expression

Timeline 1 | **The history of polyamine research**



PROTEASOMAL DEGRADATION
Degradation of proteins involving the proteasome, a 26S multiprotein complex that catalyses the breakdown of polyubiquitylated proteins. Ornithine decarboxylase is the only non-ubiquitylated protein known to be degraded by the 26S proteasome.

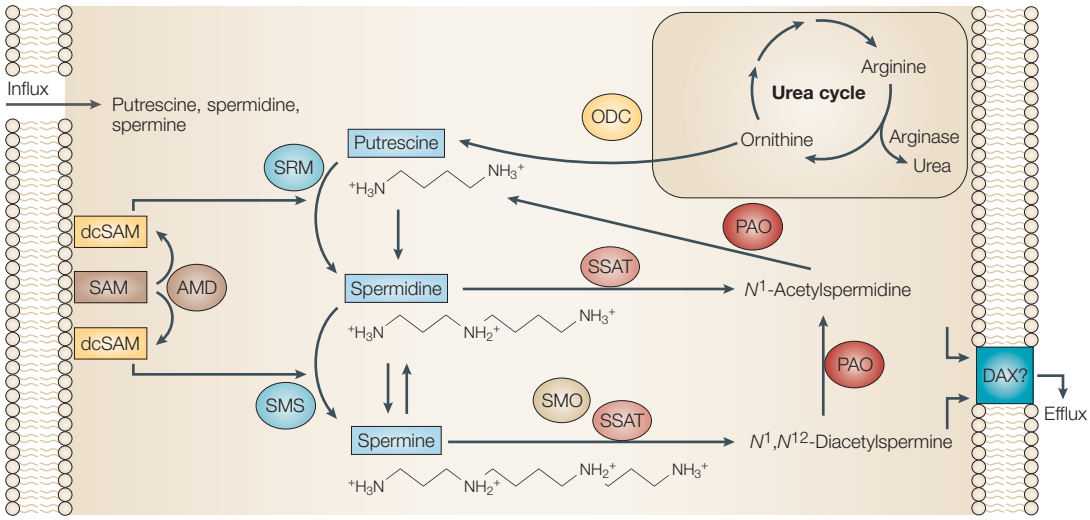


Figure 1 | **Polyamine metabolism in mammals.** The metabolism of arginine, which is produced in the urea cycle, through the action of arginase, results in the production of ornithine (other details of the urea cycle are not shown). Ornithine decarboxylase (ODC) is required for the first step in polyamine synthesis, in which ornithine is decarboxylated to produce putrescine. Decarboxylation of *S*-adenosylmethionine (SAM), by *S*-adenosylmethionine decarboxylase (AMD) yields decarboxylated SAM (dcSAM), which donates its propyl amine moiety (not shown) for the formation of spermidine and spermine by spermidine synthase (SRM) and spermine synthase (SMS), respectively. The spermidine/spermine N^1-acetyltransferase (SSAT) is a propylamine acetyltransferase that monoacetylates spermidine and can either mono- or di-acetylate spermine. These acetylated polyamines have at least two potential fates. Diamines and acetylated polyamines are substrates for export by the putative transporter DAX (diamine exporter)[116], and are then eliminated in urine[1]. Acetylated spermidine and spermine are also substrates for a flavin-dependent polyamine oxidase (PAO), which catalyses their conversion back to putrescine. A spermine oxidase (SMO), which can oxidize non-acetylated spermine, has recently been characterized[117], although its physiological role has not been established. Putrescine, spermidine and spermine can also be imported from extracellular compartments through a transport mechanism that is not well defined.

of which is associated with the development of Burkitt's lymphoma and several epithelial cancers, in addition to colorectal cancer[28]. *MYC* encodes a transcription factor that is required for the proliferation of some normal cells, but when overexpressed leads to uncontrolled growth and cancer[29]. Several groups have demonstrated that *ODC* is a direct transcriptional target of MYC[30,31]. *ODC* gene expression is increased in intestinal tissue of $Apc^{Min/+}$ mice — a model for FAP in which wild-type alleles of the murine homologue of *Apc* are lost. Furthermore, a specific inhibitor of ODC suppressed intestinal carcinogenesis in this model[32]. The hypothesis that ODC is a modifier of APC-dependent tumorigenesis is supported by subsequent work showing that conditional expression of wild-type APC suppresses *ODC* gene expression in a MYC-dependent manner in human colon tumour cells[33]. The transcriptional regulation of ODC involves other mechanisms in addition to the pathway involving APC and MYC. For example, RAS-dependent fibroblast transformation involves induction of ODC expression[34]. ODC has been shown to cooperate with RAS in skin carcinogenesis in mice[35].

ODC is not the only polyamine metabolic gene that is regulated by oncogenes and tumour-suppressor genes. The ODC regulator antizyme (OAZ), which targets ODC for PROTEASOMAL DEGRADATION, is also regulated by APC in the $Apc^{Min/+}$ mouse model. When wild-type APC is lost in intestinal epithelia of these mice, decreased OAZ activity contributes to increased ODC levels[32] (FIG. 2). In addition, expression of spermidine/spermine N^1-acetyltransferase (SSAT) is negatively regulated by the *KRAS* oncogene, which is commonly mutated — and, as a result, aberrantly activated — in human colon cancer and other gastrointestinal

Box 1 | **Polyamines in normal growth, development and tissue repair**

Polyamines have long been associated with cell proliferation, hypertrophy (increase in cell size) and tissue growth, and are known to be involved in the development of several tissue types. Substantial evidence for the functional involvement of polyamines in the normal development of the intestinal tract has accumulated. Early studies indicated that polyamine-synthesis inhibitors disrupted intestinal development in mice[91,92]. Subsequently, it was found that the repair of gastric and duodenal injury was also dependent on polyamine metabolism[93,94]. The role of polyamines in tissue repair might be to facilitate tissue remodelling, as has been reported for certain types of lung damage[95]. Inhibition of polyamine synthesis suppresses wound healing in rodents[96].

Polyamines have also been implicated in the development and function of both male[97] and female reproductive organs[98,99]. Aberrant expression of spermidine/spermine N^1-acetyltransferase influences specific gene expression controlling reproductive-tract tissue growth and function[100].

In addition, increases in polyamine metabolic-enzyme activities and tissue polyamine levels have also been associated with the normal growth and hypertrophy of several other tissues — including skin, breast, kidney and heart — in rodents[1]. However, pharmacological or genetic suppression of these enzymatic activities does not prevent either cardiac or renal hypertrophy responses[101,102]. So, polyamines are functionally involved in growth responses of some tissues, but are only associated with growth responses in others.

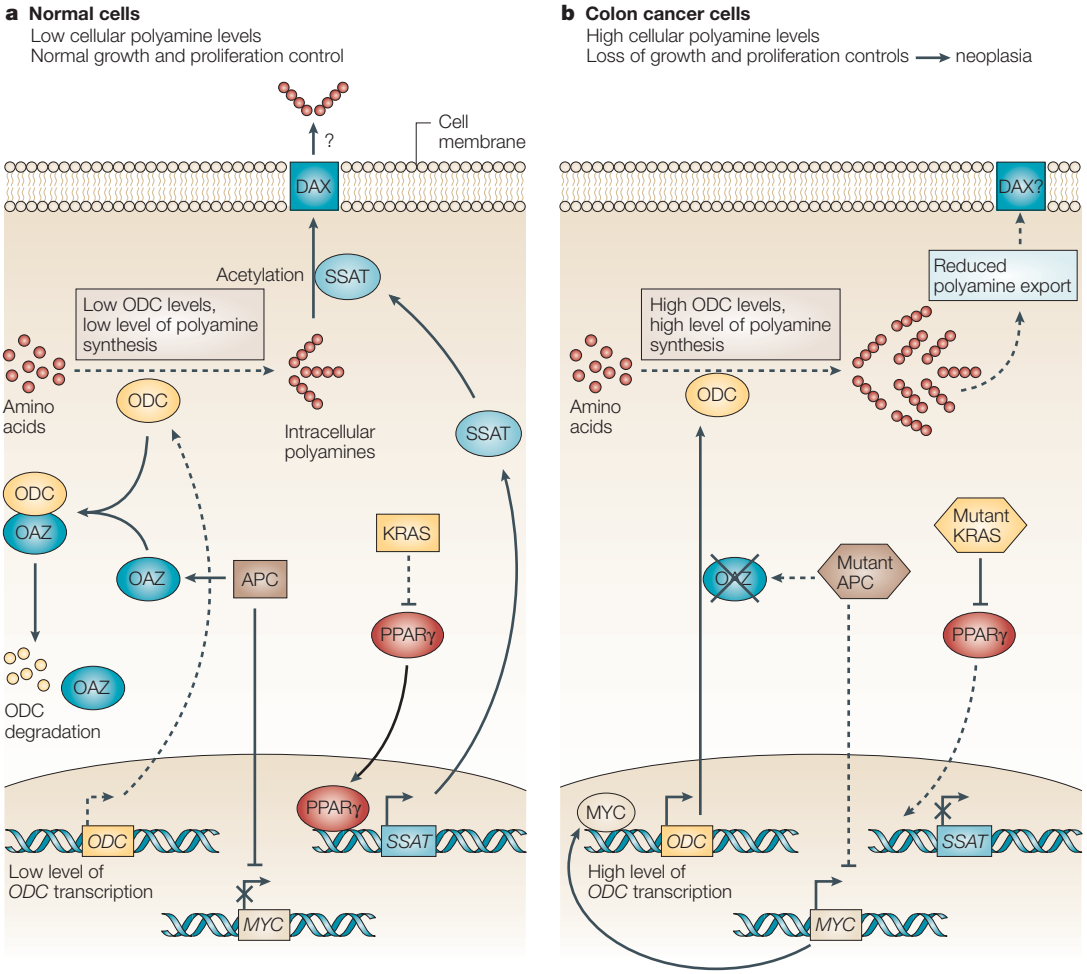


Figure 2 | **Regulation of polyamine metabolism by oncogenes and tumour-suppressor genes involved in the development of colon cancer. a** | Normally, the tumour suppressor adenomatous polyposis coli (APC) regulates polyamine synthesis in human colon tumour cells and murine and human intestinal epithelia. Cell-culture studies indicate that wild-type APC suppresses transcription of *MYC*, which is an activator of ornithine decarboxylase (*ODC*) transcription. Wild-type APC also acts to regulate ODC antizyme (OAZ), a protein that regulates ODC activity by targeting it for degradation. In normal cells and tissues, most of the KRAS protein is inactive as a signalling molecule. The tumour-suppressor peroxisome-proliferator-activated receptor-γ (PPARγ), which activates spermidine/spermine N^1-acetyltransferase (*SSAT*) transcription, is repressed by active KRAS. Therefore, in normal cells and tissues, wild-type APC and KRAS lead to reduced proliferation, increased apoptosis and reduced neoplasia. **b** | When APC is mutated or deleted, as occurs in individuals with familial adenomatous polyposis, ODC expression is increased. Loss of APC function results in a decrease in OAZ, which contributes, along with transcriptional activation as discussed above, to increased ODC activity. Oncogenic mutations in KRAS, which prevent hydrolysis of bound GTP and activate KRAS signalling activity, suppress polyamine catabolism. Consequently, mutant APC and KRAS act to promote neoplasia by increasing polyamine biosynthesis and suppressing polyamine catabolism. Increased levels of polyamines are associated with increased cell growth, decreased apoptosis and expression of genes, including some involved in tissue remodelling. These processes contribute to normal development, tissue repair and, when deregulated, neoplasia.

cancers[36]. The KRAS-dependent signalling pathway regulates *SSAT* transcription through a mechanism involving peroxisome-proliferator-activated receptor-γ (PPARγ), a putative tumour suppressor. PPARγ positively regulates the transcription of *SSAT* through a PPAR response element (*PPRE*) in the *SSAT* promoter[37]. KRAS suppresses *SSAT* transcription by inhibiting PPARγ expression and subsequent binding to the *SSAT* promoter[36]. So, polyamine synthesis and catabolism are both regulated by signalling pathways that are influenced by oncogenes and tumour-suppressor genes. These results indicate how polyamine levels become increased in colorectal and other gastrointestinal cancers.

Polyamine levels are also increased in other epithelial cancers, including skin and prostate cancers. HRAS cooperates with ODC in skin carcinogenesis, but there is as yet no evidence to implicate HRAS in the regulation of polyamine metabolic enzymes in this tissue[35]. *ODC* transcription is regulated by androgens in mammalian and human prostate cancer cells[38]. Inappropriate *MYC* expression due to chromosomal translocation is the

basis for neoplasia in Burkitt's lymphoma[29]. Inappropriate expression of MYC in models of this disease results in inappropriate *ODC* expression and tumorigenic phenotypes, including increased proliferation and decreased apoptosis[30,31]. In addition, ODC is regulated by the putative oncogene eukaryotic translation initiation factor 4E (*eIF4E*) in a range of cancers[39].

Evidence for a causative role in cancer

A recent study of genetic variability affecting *ODC* expression has provided evidence that increased polyamine synthesis and retention in the cell has a causative role in human cancer colon cancer, rather than being a purely associative effect[40]. The relationship of a SINGLE-NUCLEOTIDE POLYMORPHISM (SNP) affecting *ODC* promoter activity to risk of COLON-POLYP recurrence in a human colon-polyp prevention study was evaluated. The *ODC* SNP (G315A) occurs 315 nucleotides downstream of the *ODC* transcriptional start site and is located between two consensus E-boxes in the promoter region. The transcriptional activator MYC and the transcriptional repressor MAD1 bind to these elements. The frequency of this SNP has been measured in several groups of people, including participants in a colon cancer prevention trial. Fifty-five percent of participants in this trial, all of whom previously had a colon polyp, were homozygous G at this locus; thirty-five percent were heterozygous G/A and ten percent were homozygous A. The SNP was shown to have functional consequences for *ODC* expression. The transcriptional repressor MAD1, a MYC antagonist, selectively repressed *ODC* transcription in an A-allele-specific manner. Finally, it was found that the A-allele was associated with a statistically significant reduction in risk of colon-polyp recurrence and that this risk was even further reduced in participants who reported taking aspirin regularly. Aspirin use does not affect allele-specific transcription of *ODC*, but does affect polyamine catabolism by inducing the transcription of *SSAT* (see later).

The *ODC* A-allele therefore seems to be a potential prognostic factor for polyp risk in humans, and allele number might be a predictor of human response to aspirin as a colon cancer-preventive agent. The *ODC* A-allele favours binding of the transcriptional repressor MAD1. So, the *ODC* A-allele might work in concert with aspirin to lower tissue polyamine levels and, therefore, the risk of colon-polyp recurrence.

Polyamine functions in cancer

Polyamines affect numerous processes in carcinogenesis. Increased polyamine levels are associated with increased cell proliferation, decreased apoptosis and increased expression of genes affecting tumour invasion and metastasis. Conversely, suppression of polyamine levels is associated with decreased cell growth, increased apoptosis and decreased expression of genes affecting tumour invasion and metastasis[37,41]. These generalizations need to be placed in context. Several reports, including our own, have documented that extremely high polyamine content can cause apoptosis and consequent ulceration[42–44]. However, these cases of polyamine-induced apoptosis occur primarily when intracellular polyamine levels are abnormally high, and are a consequence of loss of regulation of polyamine homeostasis.

Polyamines are necessary for blood-vessel development (angiogenesis) occurring in response to damage to normal tissues or tumour growth. Inhibition of polyamine synthesis blocks angiogenesis in models of gastric ulceration[42] and in tumour models[45,46]. Polyamine metabolism also contributes to arginine-dependent effects on colon tumour cell growth[45,46]. Arginine is metabolized to either ornithine, requiring the enzyme arginase (FIG. 1), or nitric oxide. ODC can be inactivated by nitric oxide through nitrosylation[47]. Arginine and its catabolite NG-hydroxy-L-arginine can induce cell growth arrest by inhibiting arginase in a manner that is rescued by exogenous polyamines[48]. Presumably, inhibition of arginase prevents polyamine production both by suppressing ornithine production and by favouring nitric oxide production, which inhibits ODC.

However, although polyamines seem to be associated with numerous cellular processes, a key criticism of polyamine research has been that the specific mechanisms underlying their modes of action — including those in cancer cells — have not been defined. Over the past few years, polyamines have been shown to affect specific gene expression through both transcriptional and post-transcriptional processes (BOX 2).

cDNA microarray technologies have been used to identify polyamine-regulated genes in cancer cells. In one example, cells derived from human colon tumours were treated with DFMO. This ODC inhibitor decreased cellular putrescine and spermidine levels and also blocked the ability of these cells to form tumours in severe combined immunodeficient mice when added to drinking water[41]. DFMO suppressed the expression of several genes involved in two types of cell–cell interactions, TIGHT JUNCTIONS and GAP JUNCTIONS. Tight-junction proteins have been thought to act as tumour suppressors[49], whereas gap-junction proteins have been implicated in cell communication involved in carcinogenesis[50]. Experiments in cell culture showed that the effects of the ODC inhibitor could be rescued by exogenous putrescine, supporting the interpretation that these genes are regulated by polyamine-dependent mechanisms. Other groups have observed similar effects of DFMO on cell-junction proteins[51,52], although alterations seem to be cell-type specific.

Polyamines contribute to the regulation of other cancer-related functions, including apoptosis and proliferation. Termination of fetal development is associated with increased apoptosis in embryonic cells lacking ODC. As discussed earlier, *Odc*-knockout mice die early during embryogenesis[7]. Increased levels of polyamine have long been associated with proliferation, and recent experiments with genetically altered rodents have confirmed the important roles of these molecules in both normal and neoplastic growth[53].

SINGLE-NUCLEOTIDE POLYMORPHISMS (SNPs). Single-base-pair changes in DNA that differ among individuals.

COLON POLYP
Non-invasive but neoplastic growths that develop from normal colon mucosa and that can develop into colon cancer.

TIGHT JUNCTIONS
Intercellular junctions that act as barriers to specific tissue processes.

GAP JUNCTIONS
The most widespread type of intercellular junction, involved in coupling cells both electrically and metabolically.

Box 2 | **Mechanisms of polyamine-dependent gene expression**

Ornithine decarboxylase (ODC) and its diamine product putrescine participate in a range of cellular processes affecting cell behaviours. Several specific mechanisms influencing gene expression have been described that require unique polyamines or putrescine , which is their diamine precursor. In the early 1980s, Myung Park and collaborators demonstrated that the polyamine spermidine was the essential substrate for the post-translational modification of the putative eukaryotic translation inhibition factor 5A (eIF5A)[103]. Subsequent studies have shown that the *Saccharomyces cerevisiae* equivalent of *eIF5A* is an essential gene in this yeast[104], but is not essential for general protein synthesis[105,106]. Rather, eIF5A seems to be involved in the processing of specific RNAs[107,108].

A second example of a specific mechanism of polyamine-mediated gene expression is the formation of ODC antizyme (OAZ), a protein that targets ODC for proteasomal degradation[109]. The *OAZ* transcript includes two overlapping open reading frames. When polyamine levels are low, a functional full-length OAZ protein is not made because translation terminates at a stop codon only 65 codons downstream of the translation start site. Increased polyamine levels stimulate a translational frameshift that causes the paused RIBOSOME (paused as a consequence of a downstream 'pseudoknot') to jump reading frames and read through the first in-frame stop codon[110]. At present, OAZ is the only known mammalian protein that is influenced by polyamines in this manner. OAZ expression also influences DNA methylation and specific gene expression[111]. The specific mechanisms by which OAZ affects DNA methylation and subsequent gene expression are unknown.

As charged cations at physiological pH, polyamines have long been known to associate with nucleic acids, and numerous investigators have described phenomena, ranging from global effects on chromatin structure to effects on specific DNA–protein interactions, by which polyamines affect gene transcription[1]. Enzymes that modify the overall charge of polyamines, including polyamine acetyltransferases[112] and polyamine oxidases[113], are well known, and neutralizing the positive charges of polyamines could have a similar affect on transcription as histone acetylation, which results in the neutralization of the primary amine in lysine residues. Recently, a polyamine-oxidase-like protein has been identified as a component of a transcriptional co-repressor complex in *Caenorhabditis elegans*[114].

So, polyamines act to influence specific gene expression by both transcriptional and post-transcriptional mechanisms. Some of these mechanisms seem to be very limited (for example, the translational frameshift affecting OAZ synthesis), whereas other mechanisms, like eIF5A, affect specific sets of proteins.

Use of polyamine metabolic inhibitors

Interest in targeting polyamine metabolism as a potential strategy for cancer chemotherapy was stimulated in the early 1970s by a study from Williams-Ashman and Schenone[54]. This work indicated that methylglyoxal (bis) guanylhydrazone (MGBG), a drug used in the treatment of leukaemia, inhibited *S*-adenosylmethionine decarboxylase (AMD) — a key enzyme in polyamine synthesis (FIG. 1; TABLE 1) — and the formation of spermidine and spermine. However, MGBG applications in cancer therapy were limited because of drug-induced toxicity, especially to self-renewing normal tissues, including the bone marrow and intestinal tract.

In the mid-1970s, the Centre de Recherche Merrell International (later affiliated with The Dow Chemical Company), a pharmaceutical firm in Strasbourg, France, began a research programme to develop other, less toxic, inhibitors of polyamine metabolism and to test their efficacy in cancer therapy. Investigators at this institute demonstrated that inhibitors of ODC suppressed mammalian tumour cell growth[55]. Those findings spurred the development of a wide range of very selective ODC inhibitors, including DFMO[56]. DFMO is a specific ODC inhibitor that has proven to be effective in the treatment of certain hyperproliferative and infectious diseases, including removal of excess facial hair in women and in the treatment of African sleeping sickness[57]. The Merrell group also developed reagents that inhibited AMD[58] and the flavin-dependent polyamine oxidase (PAO), which is involved in polyamine catabolism[59]. PAO catalyses a reaction to produce the shorter-chain amines putrescine and spermidine, as an alternative to synthesis from amino acids. Some evidence exists to support the hypothesis that combinations of polyamine-synthesis inhibitors and PAO inhibitors might be more potent antiproliferative strategies than either type of inhibitor alone[60]. In addition to the development of drugs that target specific enzymes in the polyamine pathway, structural analogues of the polyamines themselves have been synthesized and evaluated as potential anticancer drugs. The rationale for this latter approach includes the fact that polyamines participate in both the negative regulation of polyamine biosynthetic enzymes and the positive regulation of polyamine catabolic enzymes. As listed in TABLE 1, Phase I (toxicity assessment) trials have recently been reported for the AMD inhibitor SAM4861 (REF. 58) and several polyamine analogues that do not specifically target a particular enzyme, but rather have features similar to the polyamines themselves. One Phase II (efficacy assessment) trial found the polyamine analogue DENSPM to be well tolerated, but without evidence of therapeutic benefit[22]. Other Phase II trials are ongoing, but results have not yet been reported.

The development of these selective inhibitors of polyamine metabolism enabled the closer examination of effects of polyamine depletion in cell culture — depletion of intracellular polyamines suppressed growth, but was not generally toxic to cells[61]. The inhibitors were also evaluated in combination with cytotoxic anticancer agents (drugs and ionizing radiation) and were found to have modest interactive effects in animal models[62]. From the late 1970s and throughout the 1980s, DFMO was actively evaluated as an anticancer drug, either alone or in combination with other agents, in clinical trials. DFMO was chosen over other ODC inhibitors in these early clinical trials for several reasons, including activity in animal models and pharmacokinetic properties in humans that allowed favourable physiologically significant concentrations of the drug in serum. DFMO, either alone or in combination with other chemotherapies or radiotherapy, was generally found to have little antitumour activity in Phase I trials. Phase II trials also showed DFMO to be generally ineffective as a treatment for patients with leukaemias and brain tumours[63]. The reason for the lack of cancer therapeutic efficacy of DFMO might be a consequence of the finding that this agent does not generally kill cells. As successful therapies are generally cytotoxic, either through direct or indirect action, lack of cancer therapeutic efficacy of DFMO is not that surprising. However, DFMO does suppress the expression of genes that are involved in cell proliferation, tissue remodelling and/or tumour invasion[41,64]. These

RIBOSOME
Particles composed of RNA and protein that are sites of protein synthesis.

Table 1 | **Inhibitors that target polyamine metabolic enzymes or mimick polyamine structures**

Inhibitor	Mode of action	Status of therapeutic trials	References
Difluoromethylornithine (DFMO)	ODC inhibitor	Therapeutic trials generally fail to show efficacy; chemoprevention trials ongoing (TABLE 2)	18,63,90
(2R,5R)-6-heptyne-2,5-diamine (MDL 72.175, MAP)	ODC inhibitor	Phase I trial completed, not developed further	118
Methylglyoxal (bis) guanylhydrazone (MGBG)	AMD inhibitor	Efficacy in trials for leukaemia, but toxic	119
4-amidinoindan-1-one 2′-amidinohydrazone (CGP 48664 or SAM486A)	AMD inhibitor	Phase I clinical trial completed, not developed further	58
N^1,N^{11}-diethylnorspermine (DENSPM)	Polyamine analogue	No apparent efficacy in Phase II trial; other Phase II trials ongoing	22
N^1,N^{14}-diethylhomospermine (DEHSPM)	Polyamine analogue	Not recommended for further study after Phase I trial, neuro- and hepatic toxicities	120

AMD, *S*-adenosylmethionine decarboxylase; ODC, ornithine decarboxylase.

features of DFMO might contribute to the potency of this drug as a cancer-preventive agent, at least in experimental models.

Use of DFMO for cancer prevention

In contrast to the rather moderate effects of DFMO on models in which cancer is already established, DFMO is a potent inhibitor of carcinogenesis. Chemical and physical carcinogenesis proceed by a series of events, including an initiation phase involving DNA damage, leading to gene mutations, followed by a promotion phase characterized by proliferation of initiated cells. Elegant studies by Weeks *et al.*[65] showed that DFMO suppressed skin carcinogenesis by blocking the promotion phase of this process. The mechanism of this effect is thought to involve inhibition of the increased cell proliferation that is associated with the promotion phase. Subsequent studies indicated that DFMO was a potent inhibitor of epithelial carcinogenesis in a range of models, including those for skin, breast and colon[18].

The activity of DFMO as an inhibitor of carcinogenesis in experimental models indicated that it might be a potent chemopreventive agent in humans. DFMO inhibits intestinal polyp formation in $Apc^{Min/+}$ mice[32]. *APC* mutations are found in almost all sporadic (non-hereditary) forms of colon cancer, and ODC levels are increased in colon polyps in humans[18]. Our group has therefore carried out chemoprevention trials with DFMO in patients with FAP and sporadic colon polyps.

The aim of these trials was to adopt the simplest possible strategy for drug delivery to maximize compliance in these individuals, who had not yet developed cancer. Serum levels of DFMO and tissue levels of polyamine were measured (BOX 3). Although the serum half-life was known to be in the order of hours, single daily oral doses of the drug were able to suppress certain tissue polyamine content[66].

ODC is also upregulated in other intraepithelial neoplasias (IENs) — non-invasive precursors of epithelial cancers. Development of DFMO as a potential chemopreventive agent for use in human diseases has been most systematically developed for colorectal cancer, non-melanoma cutaneous cancer, bladder cancer and cervical cancer, with a somewhat less aggressive approach in Barrett's oesophagus and breast cancer. A summary of the current status of definitive clinical trials and evidence supporting their undertaking is provided in TABLE 2.

Identification of DFMO toxicities. For a candidate compound to be adopted in a preventive setting, it is essential that the drug causes minimal or no toxicity[67]. This requirement is dictated by the fact that cancer prevention involves treatment of basically healthy individuals who are at risk of, cancer, but have not developed the disease. Therefore, identifying the limiting dose for DFMO — that which produces no evident side effects, but results in the desired biochemical effect in the tissue/organ under study (in this case the colon or skin) — was crucial before implementation of definitive or pivotal trials, which could support New Drug Applications to the Food and Drug Administration (FDA).

Box 3 | **Measuring polyamine variables in clinical studies**

Early in our clinical chemoprevention studies with difluoromethylornithine (DFMO), we decided that we needed to measure tissue variables that would indicate the efficacy of the treatment. We conducted a clinical evaluation of variability in a range of factors affecting tissue polyamine levels[115]. These variables included measures of ornithine decarboxylase (ODC) and spermidine/spermine N^1-acetyltransferase RNA levels and enzyme activities as factors affecting polyamine levels and direct measures of polyamine levels. This study identified several sources of error in measurements of these variables in human tissues and indicated that direct assessment of polyamine levels in target tissues using high-performance liquid chromatography methods was the most reliable measure of polyamine metabolism for predicting the effects of intervention using agents like DFMO. Assessment of the suppression of ODC enzyme activity in skin biopsy samples might also be a reliable marker of the effects of DFMO in patients undergoing treatment for skin cancer prevention[14,73]. Attempts to use an easily accessible tissue such as shed buccal mucosal cells as a surrogate for other internal organs were unsuccessful, largely because of contamination of the buccal mucosal cells by endogenous bacteria and artificial increases of polyamine levels[75].

Other important variables that have been identified include age, as both putrescine levels and the ratio of spermidine to spermine, and changes in these variables as a function of DFMO treatment, decrease as a function of donor age[66].

Table 2 | **Status of definitive or pivotal clinical chemoprevention trials with DFMO**

Cancer	Preclinical evidence for activity*	Supportive evidence from pilot/Phase I/ Phase II trials‡	Target	Definitive or pivotal randomized trials	Location§
Colorectal	Strong	Strong	Polyps	DFMO and sulindac‖; accrual 75% complete	University of California, Irvine
Colorectal	Strong	Strong	Polyps	DFMO and celecoxib‖; accrual ongoing	MD Anderson Cancer Center
Skin	Strong	Strong	Actinic keratosis	DFMO; accrual complete	University of Wisconsin
Cervical	Moderate	Moderate	Cervical intraepithelial neoplasia	DFMO; accrual ongoing	MD Anderson Cancer Center
Bladder	Moderate	Moderate	New squamous-cell carcinoma	DFMO; accrual complete	University of Rochester
Oesophageal	Moderate	None	Barrett's oesophagus	–	–
Breast	Weak	None	Surrogate markers of breast cancer¶	–	–

*Preclinical evidence includes effects on both cell and animal models of cancer[18]. ‡Supportive evidence includes effects of agents on surrogate markers of cancer development (for example, suppression of tissue polyamines) at doses that cause minimal toxicity to patients. §Further information on these chemoprevention trials can be found in the online links box. ‖Sulindac and celecoxib are both non-steroidal anti-inflammatory agents. ¶Cytology and measures of proliferation. DFMO, difluoromethylornithine.

Testing DFMO as a treatment, described above, had already identified changes in hearing as a potentially limiting toxicity in prevention trials[63]. However, a series of clinical studies by our group and investigators at Wisconsin, primarily targeting people with colon and skin IENs, have established that the hearing changes affect PURE TONE only, are rapidly reversible following discontinuation of the drug, and that a low dose of DFMO can be used that does not produce measurable changes in hearing, but nevertheless results in substantial lowering of polyamine levels and/or ODC inhibition[68–70].

These results also have implications for the use of DFMO in other organs. If polyamine levels cannot be substantially reduced in an organ at the limiting dose (about 0.5 g/m^2/day) then use of the drug in a particular chemoprevention setting might not be realistic. For example, a limiting dose of DFMO does lower polyamine levels substantially in cervical tissue, but does not do so in oral or breast tissue, and probably the bladder[9,71–77]. In many tissues, including the colonic mucosa, DFMO suppresses putrescine and spermidine, but not spermine[66,78]. In prostate tissue, which contains 5–10 times more spermine than either putrescine or spermidine, a one-month treatment with DFMO did reduce spermine levels[76]. Therefore, the effects of DFMO in specific tissues depend both on the expression of the target enzyme and the regulation of the individual amines. The expression of ODC, the target for DFMO, might not be sufficiently increased and might therefore contribute to the growth of IENs in these tissues. In addition, DFMO did not reverse Barrett's oesophagus lesions — the failure to affect either tissue polyamines or other markers might have contributed to the fact that clinical trials have not progressed for these IENs (TABLE 2).

Use of DFMO in combination with NSAIDs. Our initial colon cancer prevention trials, to assess the safety and efficacy of suppressing polyamine content in colorectal mucosa, used DFMO alone[9,71–77]. Subsequently, studies in experimental model systems demonstrated that several signalling pathways were de-regulated in colon carcinogenesis, and prevention strategies using combinations of inhibitors were more effective than single-agent strategies[40]. Specifically, combinations of DFMO and non-steroidal anti-inflammatory drugs (NSAIDs) were shown to be potent combinations in experimental models. NSAID use has been consistently associated with a reduced risk of colon cancer[79]. There are two aspects to the rationale for combining ODC inhibitors such as DFMO with NSAIDs (FIG. 3). First, cyclooxygenases (COXs) — which are inhibited by NSAIDs — are potent modifiers of APC-dependent intestinal carcinogenesis in mouse models. Genetic or pharmacological suppression of COX2 markedly reduces numbers of intestinal tumours[80], and the COX2 inhibitor celecoxib suppressed polyp size in a clinical trial of patients with FAP[81]. COX2 acts on arachidonic acid to produce prostaglandins. The specific mechanisms involved in COX2-dependent tumorigenesis are not yet fully defined, but might involve activation of gene expression mediated by cell-surface or intracellular prostaglandin receptors[82]. Some, or all, of these mechanisms might be totally independent of polyamines. Therefore, COX2 and ODC seem to act as modifiers downstream of the *APC* tumour-suppressor gene in both animal models and in humans. Cell-culture studies indicate that DFMO and the NSAID sulindac act additively to suppress colon tumour cell viability[64], indicating that these agents are acting through independent mechanisms. It is unknown whether NSAIDs can suppress tumour formation by mechanisms that are totally independent of COXs and polyamines.

The second aspect of the rationale for combining ODC inhibitors and NSAIDs is that *SSAT* is a transcriptional target for several NSAIDs. The non-selective COX1/COX2 inhibitor sulindac activates PPARγ, which recognizes a unique DNA sequence in the *SSAT* promoter to induce transcription of this

PURE TONE
A single frequency tone measured as part of clinical audiometric evaluations.

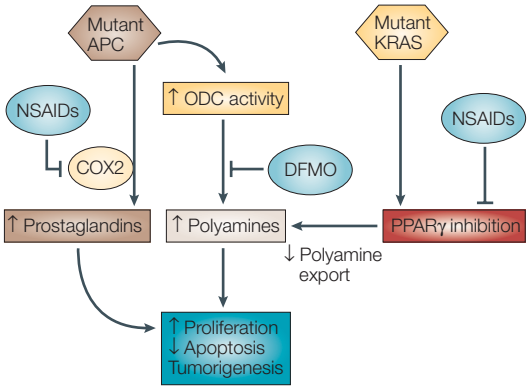


Figure 3 | **Rationale for combination chemoprevention with inhibitors of polyamine synthesis and NSAIDs.** The amino-acid-derived polyamines and the arachidonic-acid-derived prostaglandins have both been associated with increased proliferation and decreased apoptosis associated with tumorigenesis. Ornithine decarboxylase (ODC) is the first enzyme in the polyamine biosynthetic pathway and the cyclooxygenases (COXs) are involved in prostaglandin production. Mutant APC leads to increased ODC and COX2 expression. Inhibition of ODC and COX2 leads to decreased proliferation and decreased suppression of apoptosis. Although difluoromethylornithine (DFMO) is a specific inhibitor of ODC and non-ODC mechanisms of DFMO activity have not been reported, non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to exert both COX-dependent and COX-independent actions. One COX-independent action includes the activation of the polyamine catabolic enzyme spermidine/spermine N^1-acetyltransferase (not shown) and involves activation of peroxisome-proliferator-activated receptor-γ (PPARγ). Mutant KRAS suppresses PPARγ expression in colon cells. Together, DFMO and NSAIDs act to reduce neoplasia.

gene[37]. NSAIDs that are structurally unrelated to sulindac, like aspirin, also induce *SSAT* transcription[40] by both COX-dependent and -independent mechanisms[37,40]. Aspirin seems to activate *SSAT* transcription through NF-κB and AP-1 consensus elements in the *SSAT* promoter (N. Babbar, R. Casero and E. W. G., unpublished observations). One mechanism of the chemopreventive and therapeutic effects of certain NSAIDs is their ability to induce apoptosis. NSAID-induced apoptosis can be reversed, in part, by exogenous polyamines[37,83], indicating that NSAID-induced SSAT induction and subsequent polyamine export are causally involved in NSAID-induced apoptosis. Consequently, NSAIDs act as inducers of polyamine catabolism and export, and complement inhibitors of polyamine synthesis in acting to lower tissue polyamine levels. So, ODC inhibitors and NSAIDs counter the effects of mutation of tumour-suppressor genes, such as *APC* and *PPARγ*, and oncogenes, such as *MYC* and *KRAS*, which act to increase tissue polyamines in cancer. Combinations of DFMO and NSAIDs work at least additively in several models of colon and intestinal carcinogenesis[67,84], corroborating this model. It is hoped that these results of combination chemoprevention in experimental model systems will translate to use in patients with colorectal cancer.

Ongoing DFMO chemoprevention trials. The first goal of our cancer chemoprevention trials was to define a dose of DFMO that was both safe and effective. Our dose de-escalation studies and subsequent randomized placebo-controlled studies demonstrated that oral doses of DFMO between 0.25 and 0.5 g/m^2/day for times as short as 1 month and as long as 1 year were effective in reducing rectal polyamine content in humans[85–87]. These studies also demonstrated that these doses, which can reduce rectal polyamine levels, did not produce detectable toxicities in excess of those observed in the group receiving placebo.

A recently completed clinical trial assessed the toxicity of DFMO in combination with low doses of sulindac. Final toxicity results from this trial will be available within 2 years. Although follow up in this 3-year treatment trial is not yet complete, and the study is still blinded, no detectable differences in toxicities have been identified between the two study groups (F.L.M., E.W.G. and C. McLaren, unpublished observations). Based on these results, a Phase III study has been initiated to determine if DFMO plus sulindac can reduce colon-polyp recurrence. Results for this polyp-recurrence trial, which also uses a 3-year treatment duration, will be available within 5 years, taking into account time for patient accrual to the study and treatment time.

When we initiated our clinical studies, there was great concern in the medical community regarding the potential hearing-related toxicity of DFMO. In the various human epithelial tissues studied, the dose of DFMO that was effective in lowering polyamine levels or inhibiting ODC ranged from about 0.25–1.0 g/m^2/day[73,76,78]. As the dose of DFMO that produces noticeable hearing changes is about 1.0 g/m^2/day, lower doses might be both efficacious and safe.

There are several biological factors that could adversely affect the potentially beneficial long-term outcomes of DFMO use in cancer prevention. These could include amplification of ODC in some tissues, as has been reported in cell-culture models[88], or increased uptake of polyamines from bacterial flora in the intestinal lumen. Both of these mechanisms could overcome the inhibitory effects of DFMO. Additionally, unexpected long-term toxicities might emerge.

Implications and future directions

'Proof of principle' for chemoprevention of cancer in humans has been established[85–87]. However, toxicity has abrogated the widespread adoption of agents, such as retinoids, for the prevention of cervical and oral cancers[89]. Recent results with finasteride in the prevention of prostate cancer do indicate that the development of new tumours could be reduced significantly in an at-risk population. However, more high-grade tumours were detected in the finasteride treatment group, raising the concern that these might be a consequence of drug use[121]. Consequently, the future application of this drug in cancer prevention is clouded by this potential risk. By contrast, DFMO toxicity has been well studied in a systematic manner,

and side effects are unlikely to prevent its use in cancer prevention if efficacy is demonstrated.

Polyamines continue to be molecules that hold fascination for biologists, chemists, molecular biologists and clinical researchers. Although these molecules have been known for over 300 years, a mechanistic understanding of their roles in normal and disease processes has only been developed in the past 30 years (FIG. 3). The first definitive clinical trials for cancer prevention are still in progress. Several aspects of polyamine metabolism and function present numerous experimental clinical opportunities. Although this review has focused on the promise of targeting polyamine metabolism in cancer prevention, increasing understanding of the role of these molecules in human cancer might lead to new ways of using these agents for cancer therapy. In this regard, a recent report indicates that DFMO might improve survival in certain patients with brain tumours[90].

Our understanding of the upstream regulation of ODC has increased markedly in the past few years and the roles of APC, MYC and related transcriptional activators and repressors offer unique opportunities for intervention. Similarly, the downstream function of polyamines and their important roles in angiogenesis and invasion have recently been identified (discussed earlier). New information relating to the role of the polyamines in normal growth, development and tissue repair, and how these processes go awry in cancer, could be manipulated for future therapeutic benefit.

The concept of combination therapy for chemoprevention is important, as combinations of agents are generally more effective than single agents in animal models. Polyamine levels can increase because of synthesis and/or uptake, and their metabolism is highly regulated by a series of catabolic enzymes in mammals. Consequently, it should not be surprising that attaining the goal of reducing high polyamine levels in tissues during cancer development might require targeting two or more of these processes. The finding that the polymorphism affecting *ODC*-promoter activity was most profoundly associated with colon-polyp recurrence in individuals taking aspirin (an activator of polyamine catabolism), indicates that interventions at several points in polyamine metabolism might be necessary to optimally repress the development of epithelial cancers. We have focused on NSAIDs as the agents used in clinical prevention trials in combination with DFMO. However, targeting features of polyamine metabolism, such as polyamine uptake and efflux, and/or catabolism, with DFMO or other polyamine-synthesis inhibitors might also be useful strategies for the prevention of epithelial cancers.

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Competing interests statement

The authors declare no competing financial interests.

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