

Rodent Models of Colon Carcinogenesis for the Study of Chemopreventive Activity of Natural Products

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Abstract

This perspective describes some commonly used animal models for the evaluation of potential chemopreventive agents in colon carcinogenesis. Special emphasis is given to the azoxymethane (AOM)/1,2-dimethylhydrazine (DMH) rat model and *ApcMin* mice, carrying a mutation in *Apc*, a key gene in human carcinogenesis. In the AOM/DMH model, colon cancers are induced chemically by high dosages of carcinogen and tumours develop mainly in the colon through a multistep

process similar to that observed in human carcinogenesis. In the *ApcMin* mice, carcinogenesis is spontaneous with no need of carcinogen administration but tumours develop mostly in the small intestine while colon tumours are less frequent. Moreover, the easy identification of preneoplastic lesions, such as aberrant crypt foci (ACF) and mucin depleted foci (MDF), in short-term studies make the AOM/DMH model a useful test for screening the potential chemopreventive efficacy of natural products.

Introduction

Colon cancer ranks second among the leading causes of cancer death in Europe [1]. Although new chemotherapy agents have improved treatment of metastatic patients, chemotherapy is not fully successful, probably because of the complex alterations present in advanced tumours. Therefore, the primary prevention of colon cancer, with approaches such as detection of precancerous lesions or individuation of risk factors is very important. In particular, chemoprevention, a strategy designed to block, reverse or delay carcinogenesis prior to tumour invasion using pharmacological or nutritional agents [2], has attracted much interest both at clinical and experimental levels. Accordingly, during the past decades, several groups of chemicals (both naturally occurring as well as synthetic) have been studied in terms of their potential chemopreventive role in colorectal cancer development. Ideally, once identified, proven safe and effective, these natural products could be included as supplements in the diet so as to reduce or slow colon cancer development. It is therefore important to rely on adequate experimental models able to identify the best candidates for further development.

In this perspective, we will illustrate some of the most used animal models for evaluation of the potential chemopreventive effects of different agents in colon carcinogenesis. To better understand the advantages and limitations of the different models examined, we will first describe the current view on colon cancer pathogenesis.

Colon Cancer Pathogenesis

Colon carcinogenesis is a multistep process starting from normal colonic cells which acquire mutations in relevant genes (oncogenes and tumour suppressors) as a result of unrepaired DNA damage from genotoxic insults. These mutated cells can then grow into preneoplastic lesions, benign tumours (adenomas) and eventually carcinomas able to invade and metastasize other tissues. This histological progression is accompanied by genetic and epigenetic alterations conferring a selective growth advantage to the progressing lesions. Most colon cancers are sporadic with only a few types having a familial basis, such as the familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) which accounts for about 5% of all colorectal cancers [3]. FAP, characterized at a clinical

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level by the development in early life of hundreds to thousands of adenomatous polyps in the colorectum, shows a dominant inheritance pattern due to mutations in the *Adenomatous Polyposis Coli* gene (*APC*) [4]. The exon 15 of the *APC* gene comprises more than 75% of the entire coding sequence (8535 bp) and is the most common target for both germline and somatic mutations. The majority of germline mutations are nonsense or frameshift mutations resulting in a truncated protein [4], [5]. HNPCC has been associated with germline mutations of the mismatch repair system genes (*MLH1*, *MSH2*, *MSH6* and *PMS2*) causing inefficient repair of the proofreading activity of DNA polymerase on short repetitive nucleotide sequences which are then expanded or contracted during DNA replication, leading to so called microsatellite instability [6], [7].

The identification of the genetic alterations causing these hereditary syndromes (FAP and HNPCC) has been instrumental to the understanding of the genetic alterations present in sporadic colorectal carcinogenesis, currently described according to the *adenoma-carcinoma* pathway, which was originally mapped by Fearon and Vogelstein [8]. In this model, colorectal carcinogenesis is caused by the accumulation of mutations in various genes, with *APC* mutations intervening in the early steps, followed by mutations in genes such as *K-RAS*, *TP53* and loss of heterozygosity (LOH) at different loci (e.g., 5q, 17p and 18q) associated with chromosomal instability (CIN), frequently found in most colorectal tumours [5]. However, further studies have demonstrated that mutations in the aforementioned genes are not inevitable [9]. For example, a realistic estimate of the frequency of sporadic tumours with *APC* mutations is probably around 50–70% [9], [10] and probably a lower percentage of colon cancers show contemporary mutations in the three genes (*APC*, *K-RAS* and *TP53*) [11]. Since *APC* is a member of the Wnt signalling, a key pathway in colon carcinogenesis, it is not surprising that mutations in other members of this pathway could produce similar molecular derangements as those caused by *APC* mutations. Accordingly, mutations in the *CTNNB1* gene (coding for the β -catenin protein, a key component of Wnt signalling), are also observed in sporadic colorectal cancers, although not frequently [12]. It has also been demonstrated that a small part of sporadic cancers carries mutations in mismatch repair systems genes leading to microsatellite instability rather than chromosomal instability [5].

Experimental Models for the Study of Colon Carcinogenesis and Chemopreventive Compounds

Several experimental models are available for the study of colon carcinogenesis such as colon cancer cell lines [13] or animal models. Rodent models have been particularly useful for *in vivo* study of the development of colon cancer and especially the che-

mopreventive activity of various pharmacological or natural products administered *in vivo*. Rodent models of colon carcinogenesis can be broadly divided into genetic models in which carcinogenesis arises spontaneously and models in which carcinogenesis is induced chemically (● Table 1).

Genetic models

Given the importance of the *Apc* gene in colorectal carcinogenesis, mouse models harbouring mutations in this gene have received great attention [14], [15], [16], [17]. The first of these genetically modified models has been the multiple intestinal neoplasia (Min) mouse (*ApcMin*) carrying a truncation mutation at codon 850 in the *APC* homologue [14]. *ApcMin* mice (C57BL6/J strain) spontaneously develop small intestinal tumours (about 30 polyps per mouse), which causes anaemia and cachexia leading to a short life span (average life span is about 120 days) [14], [18]. *ApcMin* mice have been widely used in chemoprevention experiments which are usually carried out in 1-month-old animals when tumours may be already present [19]. The effect of treatment is evaluated in control and treated animals determining tumours. However, colon tumours in *ApcMin* mice develop at a much lower frequency than in the small intestine, and since the effect of diet on colon carcinogenesis has been explained, at least in part, with variations in the luminal content of the colon (quite different from that in the small intestine), it has been argued that *ApcMin* mice are not always able to predict the efficacy of dietary chemopreventive agents in humans [20]. Additional mouse models with *Apc* mutations have also been generated such as the *Apc*^{Δ716} mice, carrying a targeted truncated mutation at codon 716 [21]. *Apc*^{Δ716} mice are similar to *ApcMin* mice regarding tumour localisation (i.e., prevalently in the small intestine) but they develop more adenomas than *ApcMin* mice and lack extra-intestinal manifestations [15], [16], [18], [21]. The mouse strain carrying a mutation at codon 1638N shows an attenuated intestinal tumour phenotype (5–6 adenoma/carcinoma), but more extra-intestinal manifestations than *ApcMin* mice [15]. Although less frequently than *ApcMin* mice, these and other additional *Apc*-based mouse models have also been used to test chemopreventive treatments [19]. *Apc*-mutant mice with a distribution of tumours more similar to that found in FAP patients would be a major advancement for chemopreventive studies. In this regard, it is interesting to note that an increase in colonic tumours has been reported in carcinogen induced *ApcMin* mice [22], [23], [24] and in *Apc*-mutant mice carrying additional specific mutations [17], [25]. Moreover, the same authors who generated the *ApcMin* mouse model have recently established a mutagen-induced nonsense allele of the rat *Apc* gene on an inbred F344/NTac (F344) genetic background (Pirc: polyposis in the rat colon) [26]. Carriers of this mutant allele develop multiple neoplasms distributed between the colon

Table 1 Major features of the most commonly used rodent models of colon carcinogenesis for the study of chemoprevention

Models	Advantages	Disadvantages
Genetic Models: <i>ApcMin</i> mice	Carcinogenesis is spontaneous with no need of carcinogen administration. Carcinogenesis is driven by a mutation in the <i>Apc</i> gene, a key gene in human carcinogenesis.	Cancers arise predominantly in the small intestine and not in the colon.
Chemically-induced carcinogenesis: AOM/DMH rat model	Carcinogenesis develops through a multistep process similar to human carcinogenesis. Cancers arise preferentially in the colon. Preneoplastic lesions easily assessed.	Cancers are induced with high dosages of carcinogens which do not represent a major source of human exposure.

and small intestine which closely resemble that found in FAP patients. Since these animals live at least 17 months, they could be particularly suitable for testing the effect of chemopreventive agents with a long-term administration regimen.

Carcinogen-induced models

Rats, like humans, spontaneously develop epithelial tumours, but colon cancer is not frequent in rats or in mice which are more prone to developing cancers in the mesenchymal tissues (like lymphomas and sarcomas) [27]. Therefore, in both rats and mice, colon cancer needs to be initiated by exogenous agents. 1,2-Dimethylhydrazine (DMH) and its metabolite azoxymethane (AOM) are the most commonly used carcinogens for inducing colon cancer (AOM/DMH model). Although the exposure to AOM or DMH is probably not relevant for human carcinogenesis and both chemicals have to be used at relatively high doses, these carcinogens induce tumours through a multistep process similar to that observed in human colorectal cancer (● Fig. 1). Interestingly, many genetic and molecular alterations found in human colon carcinogenesis such as alterations in the Wnt signalling or *K-RAS* mutations, are also found in AOM/DMH tumours [28], [29], [30], [31].

The most commonly used mice strains are CF1, CD1, C57Bl/6J, ICR, SW, Balb/c while the most frequently used rat strains are Wistar, Sprague-Dawley and the inbred strain F344 [32]. Both DMH and AOM are relatively stable chemicals and can produce a high yield of colon cancers. DMH is cheaper than AOM, but it can be more hazardous for the operators since its metabolite azomethane, a precursor of AOM, is volatile and is excreted with breathing [33]. Due to a low LD₅₀ value in mice (approximately 35 mg/kg for DMH) [34], a low dosage administered repeatedly (multiple injections) is necessary to obtain a high tumour incidence and keep mortality low [32]. On the contrary, rats have a higher LD₅₀ (about 215 mg/kg for DMH) [34] and two subcutaneous injections, one week apart, result in a good incidence and tumour yield. In our laboratory, we use two DMH *s.c.* injections (150 mg/kg one week apart in male F344 rats), obtaining a nearly 100% incidence of colonic cancers and about 1–2 colon tumours/animal (adenomas and adenocarcinomas *in situ*), when animals are sacrificed 28 weeks after carcinogen induction [29]. Both AOM and DMH induce the majority of cancers

in the colon, but tumours in the small intestine and in the inner ear are also induced, though at a lower frequency. Single AOM or DMH injections in rats have also been used but with low tumour incidence and long latency [35], [36]. In general, different tumour outcome and histology (adenomas vs. carcinomas) are obtained depending on the type and total carcinogen dose, times of injection, strain used and period of latency [32].

A typical experiment of chemoprevention in this model compares the incidence and multiplicity of tumours in two groups of AOM/DMH-induced animals, one of which has been treated with the putative chemopreventive compound. Typically, 20–30 animals are allocated to each group (controls and treated) and the effect of putative chemopreventive agents is evaluated after at least 7–9 months when tumours are developed. Treated animals are fed with the same diet as controls but supplemented with the purified molecule (e.g., polyphenols derived from fruits or a chemical drug). If the putative chemopreventive treatment is a non-purified mixture, for instance, a lyophilized vegetable extract which also contains macronutrients (carbohydrates, proteins, fibres), the experimental diets should be balanced to contain the same amount of macronutrients in both control and treated groups [37]. The putative chemopreventive agent can be administered during the various phases of carcinogenesis: before induction with AOM/DMH, during or after induction, during the promotion–progression phase of carcinogenesis. Chemopreventive molecules interfering with the metabolic activation of AOM/DMH (both molecules are procarcinogens) are defined as blocking agents of tumour initiation [38]. For instance, agents that affect CYP2E1 activity *in vivo* can modify the metabolism of the carcinogen AOM and ultimately its toxic effect [39]. On the other hand, agents with chemopreventive effect acting after the initiation phase (i.e., in the promotion or progression phases) could act as suppressing agents. When a new agent with no predictable mechanism of action is tested for its putative chemopreventive effect, it is usually administered in all the phases of the carcinogenesis process. If it has chemopreventive activity, further experiments are necessary to establish whether it is a blocking or a suppressive agent.

At sacrifice, the colon and small intestine are excised and immediately analysed for the presence of suspected tumours. Each suspected lesion is measured with a caliper and its localisation

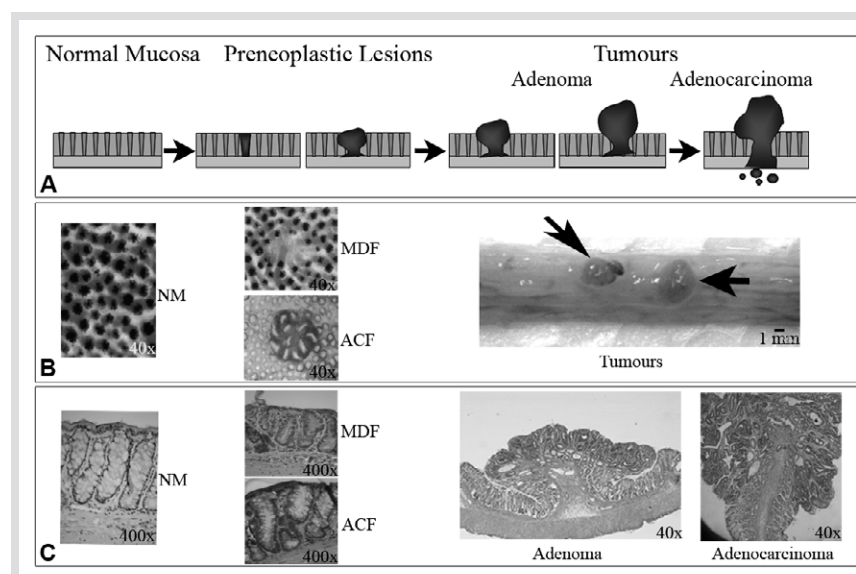


Fig. 1 The multistep process of colon carcinogenesis as observed in AOM/DMH induced F344 rats. Panel **A** shows a scheme of the process with preneoplastic lesions arising from normal mucosa and then growing into full tumours (adenomas and adenocarcinomas). In panel **B** the same lesions schematized in panel **A** as they appear in a whole unsectioned colon: preneoplastic lesions ACF and MDF can be easily observed under the microscope (original magnification as indicated in each microscopic photograph) after staining of the colon with methylene blue or HID-AB, respectively; macroscopic tumours (arrows) are observed at sacrifice. These same lesions can then be analysed more thoroughly at histology (panel **C**) to determine their dysplasia and cancer histological type (original magnification as indicated in each microscopic photograph).

along the intestine is registered (small intestine or colon and location in the colon: distal, medial or proximal). All major organs are macroscopically examined for the presence of suspected tumours or other pathological lesions. Tissues showing a deviation from normal morphology are fixed in 10% buffered formalin and embedded in paraffin blocks. If possible, due to the dimensions of the tumour, part of the lesion and possibly a piece of apparently normal mucosa could be kept frozen at -80°C or in *RNAlater*TM (Qiagen) for subsequent molecular analysis. Paraffin blocks are sectioned and stained with haematoxylin-eosin to confirm the presence and type of tumours by histopathological examination, which is performed by a pathologist unaware of the codes of the specimens. Cancer histological types are evaluated on the basis of the histotype, grading and pattern of growth [40]. Adenomas are classified on the basis of their microscopic architecture as tubular, tubulovillous and villous according to Day et al. [40].

Besides AOM/DMH, other chemical carcinogens induce colon cancer in rodents, such as some heterocyclic amines (HCA), methylnitrosourea (MNU) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). HCA such as 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) are food-borne carcinogens [41]. PhIP induces colon cancer in about 50% of male F344 rats (ACI strains are more resistant) after continuous feeding (52 weeks) of a diet containing 400 ppm; however, no colon carcinomas are observed after 2 years with 25 ppm. Compared with AOM/DMH, PhIP is more expensive and requires a chronic administration in the diet to induce cancer [42]. Therefore, although PhIP represents a more natural source of exposure to carcinogens than AOM or DMH [41], [42], it is not practical for use in routine chemopreventive experiments. Other carcinogens which have been used in chemopreventive studies are the nitrosamines methylnitrosourea (MNU) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). It has been reported that instillation of 4 successive intrarectal deposits of MNNG (0.5 mL of a solution 5 mg/mL of MNNG) twice a week for two weeks produces 80% incidence and 1–2 tumours/animal (Wistar rats) [43].

Animal models of colon carcinogenesis associated with inflammation

Inflammatory bowel diseases (IBD), which comprise Crohn's disease and ulcerative colitis, are characterised by chronic, relapsing inflammation of the intestine. One of the major risks in ulcerative colitis is the development of colorectal cancer with a 2–8-fold relative risk compared to the general population [44]. Since the efficacy of surveillance programs for individuals with ulcerative colitis remains controversial, chemoprevention, especially with natural products may be a useful strategy for reducing the risk of colon cancer in these patients. Colon cancers arising in the setting of ulcerative colitis share many of the molecular alterations found in sporadic colon cancer; however, the timing of these events seems different [44], [45], hence the need for appropriate models of colon carcinogenesis associated with inflammation to test the efficacy of potential chemopreventive agents.

Different animal models of colitis-induced colon carcinogenesis have been developed which rely on dextrane sodium sulfate (DSS; 36 000–50 000 MW) to induce inflammation (colitis) [44]. Cycles of DSS treatment mimic human ulcerative colitis; however, chemoprevention studies require large numbers of an-

imals and long periods for tumour development. Treatment of mice with the colon carcinogen AOM prior to DSS treatment (AOM-DSS model) accelerates the development of colon tumours and results in a 100% incidence of colon tumours [46]. These carcinogenesis models have been recently reviewed by Clapper and colleagues [44] and Neufert and colleagues [47]. Few chemoprevention studies have been carried out so far with the AOM-DSS model; natural compounds such as prenyloxycompounds, secondary metabolites found in plants of the Rutaceae (i.e., orange, lemon, etc.) and Umbelliferae (i.e., carrots, fennel, etc.) show promising effects [48].

Preneoplastic Lesions in Colon Carcinogenesis

Tumours are the best endpoints for evaluation of the chemopreventive effects of natural or pharmacological agents; however, since long-term carcinogenesis experiments are time and animal consuming, much effort has been dedicated to the identification of alternative endpoints correlated with carcinogenesis that can be determined at an earlier time-point. Preneoplastic lesions, representing an early step in the development of a tumour, are the ideal endpoint to be used as biomarkers in short-term carcinogenesis studies, especially if these lesions are easily identifiable in the whole colon. In 1987, Bird first described foci of aberrant crypts (aberrant crypt foci: ACF), identifiable in whole mount preparations of unsectioned colons of rodents treated with specific colon carcinogens [49]. ACF have also been identified in humans at high risk (carcinomas, adenomas, familial adenomatous polyposis) [50]. Histological analysis showed that some ACF possess typical cytological and histological features of dysplastic lesions, while others are hyperplastic lesions [51]. Genetic alterations such as increased expression of oncogenes, tumour suppressor gene mutations and microsatellite instability, have also been reported in ACF [50], suggesting that these lesions represent one of the first steps in the colon carcinogenesis process. For these reasons, and for their easy identification, ACF have been widely used as a surrogate biomarker of colon carcinogenesis [32] (► Fig. 1). In the AOM/DMH model, two s.c. injections of AOM (15 mg/kg b.w. one week apart) result in the development of 150–200 ACF throughout the colon after 2–3 months. This protocol is particularly suitable for screening potentially chemopreventive agents since it requires a relatively short duration and a smaller number of animals (about 10 rats/group) than long-term carcinogenesis experiments (► Table 2). In many chemopreventive studies, ACF results show a good correlation with long-term carcinogenesis experiments [32]; however, some studies also documented a disagreement between ACF and tumours [52], [53], probably due to the heterogeneous nature of ACF [54].

In the last decade, much effort has been dedicated to the identification of preneoplastic lesions which are better correlated with tumours than ACF. Of these, the premalignant lesions named β -catenin accumulated crypts (BCAC) described in AOM-treated rats, are dysplastic and defective in β -catenin, a transcriptional activator frequently altered in colorectal carcinogenesis [30]. BCAC identification is based on immunohistochemical techniques which do not permit an easy evaluation of the entire unsectioned mucosal surface (► Table 2); however, there are some reports of BCAC being used as endpoints in chemopreventive studies [55], [56]. Similarly, dysplastic ACF described by other authors [23], [54], [57] represent preneoplastic lesions, but their

Table 2 Features of preneoplastic lesions used as endpoints in chemoprevention studies

Lesion	Advantages	Disadvantages
ACF	Easy detection. Detected few weeks after induction. According to PubMed*, there are more than 800 papers in which ACF have been used as cancer biomarkers.	Heterogeneous population of lesions comprising both hyperplastic and dysplastic lesions. Correlation with carcinogenesis not always straightforward.
MDF	Easy detection. Dysplastic lesions. Correlated with carcinogenesis.	Low yield of lesions. According to PubMed*, there are only 15 papers in which MDF have been used as cancer biomarkers.
BCAC	Dysplastic lesions. Correlated with carcinogenesis.	Need immunohistochemistry to be detected. According to PubMed*, there are only 20 papers in which BCAC have been used as cancer biomarkers.

* PubMed (www.pubmed.gov).

identification and quantification in the unsectioned colon are problematic. Recently, we identified new lesions in the colon of rats treated with AOM, formed by crypts characterised by the absence or scant production of mucus (mucin-depleted foci, MDF) [58]. MDF are easy to quantify in the entire unsectioned colon (● Fig. 1) stained with high-iron diamine alcian blue (HID-AB) and show clear features of dysplasia [58]. The number of MDF/colon increases in rats treated with promoters of colon carcinogenesis, such as cholic acid, while it is decreased by chemopreventive agents [59]. MDF are dose-dependently induced by DMH and progressively increase in size after carcinogen administration [29]. We showed that MDF carry alterations in the Wnt signalling pathway and mutations in the β -catenin, *Apc* and *K-ras* genes, with a frequency similar to that observed in tumours [29], [31], [60]. Therefore, although MDF have been used so far in a limited number of studies [59], [61], [62], [63], [64] they are a promising biomarker for the study of the effect of chemopreventive agents in colon carcinogenesis (● Table 2).

Conclusions

Identification of natural products with chemopreventive activity in colon carcinogenesis may prove to be a useful strategy for reducing colon cancer. Before proposing natural products for human consumption their efficacy (and safety) must first be proved in animal models mimicking human colon carcinogenesis. The most commonly used animal models in colon cancer chemoprevention experiments are the AOM/DMH rat model and *ApcMin* mice, both of which have advantages and disadvantages (● Table 1). The easy identification of preneoplastic lesions, such as ACF and MDF, in short-term studies make the AOM/DMH model a useful test for screening the potential chemopreventive efficacy of many natural products.

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