



Suppression of intestinal polyposis in $Apc^{\text{min}/+}$ mice by targeting the nitric oxide or poly(ADP-ribose) pathways

Jon G. Mabley^{a,*}, Pál Pacher^a, Peter Bai^{a,b}, Rebecca Wallace^a,
Sunali Goonesekera^a, Laszlo Virag^b, Garry J. Southan^a, Csaba Szabó^a

^a Inotek Pharmaceuticals Corporation, 100 Cummings Center, Suite 419E, Beverly, MA 01915, USA

^b Department of Medical Chemistry, University of Debrecen, Debrecen, Hungary

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Abstract

Min mice have a germ-line nonsense mutation at codon 850 of the adenomatous polyposis coli (*Apc*) gene. These mice spontaneously develop multiple polyps in the small and large intestine at the age of 10–12 weeks. The aim of this study was to assess the role of reactive nitrogen species and poly(ADP-ribose) synthetase in tumorigenesis. Oxidative stress was found to be increased in the mucosa of the small intestine of $Apc^{\text{min}/+}$ mice with a concomitant increase in intestinal polyposis over control mice. Pharmacological inhibition of inducible nitric oxide synthase (NOS) with guanidinoethyldisulfide (GED) or stimulation of the breakdown of the nitrogen reactive species peroxynitrite using a potent decomposition catalyst, FP 15, reduced both the intestinal tumor load and the oxidative stress associated with intestinal polyposis in $Apc^{\text{min}/+}$ mice. Surprisingly, pharmacological inhibition of poly(ADP-ribose) synthetase by the phenanthridinone derivative PJ 34 also reduced the intestinal polyposis and oxidative stress in these mice, possibly through the inhibition of induction of nitric oxide synthase. These results suggest that reactive nitrogen species particularly peroxynitrite play a pivotal role in development of intestinal polyposis and that strategies to reduce both the oxidative stress and the formation of these radical species may be potential chemopreventive approaches for colorectal cancers.

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1. Introduction

Min mice have a germ-line nonsense mutation at codon 850 of the adenomatous polyposis coli (*Apc*) gene [1]. These mice spontaneously develop multiple polyps in the small and large intestine at the age of 10–12 weeks [1]. These mice, therefore, represent a very useful experimental model for testing experimen-

tal therapeutic interventions for the human familial adenomatous polyposis (FAP).

Nitric oxide is a reactive free radical implicated in many inflammatory processes as well as being physiologically important in the cardiovascular system along with being an important signaling molecule in most body tissues [2]. Nitric oxide is synthesized from arginine by the enzyme nitric oxide synthase (NOS). There are three isoforms of NOS; eNOS (endothelial), and nNOS (neuronal), which are both constitutively expressed, and iNOS (inducible) which when induced can result in large amounts of nitric oxide and subse-

* Corresponding author. Tel.: +1-978-232-9660;

fax: +1-978-232-8975.

E-mail address: jmabley@inotekcorp.com (J.G. Mabley).

quent tissue damage [3,4]. Potential mechanisms of tumor promotion by nitric oxide include DNA and tissue damage plus gene mutation induced by reactive nitrogen species such as peroxynitrite, formed by the reaction of nitric oxide with superoxide [5]. Peroxynitrite has been suggested to be the dominant damaging agent resulting from overproduction of nitric oxide [6]. DNA damage induced by nitric oxide or peroxynitrite leads to activation of the nuclear enzyme poly(ADP-ribose) synthetase (also known as poly(ADP-ribose) polymerase or PARP), an enzyme, the activation of which has been implicated in the pathogenesis of many inflammatory conditions [7]. Nitric oxide can stimulate tumor angiogenesis and vascular permeability in solid tumors [8]. Increased expression of iNOS has been demonstrated in ulcerative colitis [9], colon adenomas [10] and carcinomas [11] in human subjects. Nitric oxide and peroxynitrite can both activate the enzyme cyclooxygenase-2 (COX-2), which plays a critical role in the pathogenesis of a variety of cancers via prostaglandin synthesis and angiogenesis [12,13].

In this study we have investigated the effects of a specific inhibitor of iNOS, guanidinoethylidulfide (GED) [14–17], the peroxynitrite decomposition catalyst, FP 15 [18–21] and the specific poly(ADP-ribose) synthetase (PARP) inhibitor, *N*-(6-oxo-5,6-dihydrophenanthridin-2-yl)-*N,N*-dimethylacetamide hydrochloride (PJ 34) [22–26] on the development of intestinal polyposis in the *Apc*^{min/+} mouse.

2. Materials and methods

2.1. Animals

Male C57BL/6J-*Apc*^{min} and their appropriate control were obtained from the Jackson Laboratory (Bar Harbor, ME). The mice were bought at 4 weeks of age and allowed to acclimatize for 1 week at Inotek Pharmaceuticals' animal facility. Mice were maintained on the NIH 31M 11% fat diet (supplied by Purina Mills, St Louis, MI stock number 5K20). At 5 weeks of age the mice were treated orally by gavage with GED, FP 15 or PJ 34 at various doses with the dose being divided equally and given twice a day, all the compounds were prepared in water with the control animals receiving a similar volume of vehicle by gavage. The doses of drugs administered were

chosen based on previous work demonstrating effectiveness with these agents in a variety of animal models [14,16,18,19,24,27,28]. The animals were treated for 8 weeks until the mice were 12 weeks of age when they were sacrificed. In a separate set of experiments the start of treatment of C57BL/6J-*Apc*^{min} mice with GED (30 mg/(kg day)) was delayed until the mice were 8 weeks old and treated for 4 weeks until 12 weeks of age.

All animal experiments were carried out in accordance with the guidelines published by the NIH in "Principles of laboratory animal care" (NIH publication no. 85–23, revised 1985) and with the approval of the local Institutional Animal Care and Use committee.

2.2. Polyp number scoring

Immediately after sacrifice the small and large intestine was removed and opened longitudinally. The digesta was washed from the mucosal surface with phosphate buffered saline and the small intestine split into proximal, middle and distal. The numbers of polyps in each section of the small intestine and the colon were counted under a dissecting microscope (20× magnification) by a blinded investigator. One hundred percent of the mice were found to have intestinal polyps, including all mice in the experimental treatment groups ($n = 10$ – 20). Following polyp counting a biopsy of the small intestine from every animal was taken and frozen in liquid nitrogen for subsequent biochemical analysis. To reflect the profile of the whole small intestine a piece of tissue was taken from the proximal, middle and distal region of the small intestine and combined for further biochemical analysis.

2.3. Malondialdehyde assay (MDA)

Malondialdehyde formation was utilized to quantify the lipid peroxidation in the small intestine and measured as thiobarbituric acid-reactive material [29,30]. Tissues were homogenized (100 mg/ml) in 1.15% KCl buffer. Two hundred microliters of the homogenates were then added to a reaction mixture consisting of 1.5 ml 0.8% thiobarbituric acid, 200 μ l 8.1% sodium dodecyl sulfate, 1.5 ml 20% acetic acid (pH 3.5) and 600 μ l distilled H₂O. The mixture was then heated at 90 °C for 45 min. After cooling to room temperature,

the samples were cleared by centrifugation (10,000 × g, 10 min) and their absorbance measured at 532 nm, using 1,1,3,3-tetramethoxypropane as an external standard. The level of lipid peroxides was expressed as nmol MDA/mg protein, which was determined using the Bradford assay [31].

2.4. Metalloproteinase zymography

Gut samples from mice were homogenized in TNC buffer (50 mM Tris, 0.15 mM NaCl, 10 mM CaCl₂, 0.05% Brij 35, 0.02% NaN₃, pH 7.4) and cellular debris was removed by centrifugation [26,32]. Protein content was assayed by Bradfords method and samples were mixed with equal volume of 2× SDS sample buffer (Invitrogen, Carlsbad, CA). Samples were incubated at room temperature for 15 min and were applied to gelatin or casein zymography gels (3 μg). After electrophoresis (125 V, 90 min) proteins were renatured in zymography renaturing buffer (Invitrogen) for 30 min at room temperature under continuous shaking and were then placed to 37 °C for overnight developing in Developing buffer (Invitrogen). Undigested substrate was visualized by Coomassie Brilliant Blue staining (0.1% Coomassie Brilliant Blue, 45.5% methanol, 9% acetic acid). To confirm that digested bands are due to Ca²⁺ dependent proteases, replicate

gels were developed in Ca²⁺ free buffer containing 20 mM EDTA.

2.5. Statistical analysis

The results are presented as mean ± SEM; statistical analysis was performed using one-way ANOVA followed by Student–Newman–Keuls multiple comparisons post-hoc analysis, with a *P* value of less than 0.05 considered significant.

3. Results

There was a 100% incidence of intestinal polyps in the vehicle treated *Apc*^{min/+} mice as compared to 0% incidence in the control mice (Table 1). This intestinal polyposis was associated with an increase in intestinal lipid peroxidation as determined by tissue MDA levels (Table 2). Mice gut extracts were subjected to MMP zymography (Fig. 1). Gut extracts of C57BL/6 mice with no interruption in the adenomatous polyposis coli (*Apc*) gene were used as a control. The calcium dependence of the proteinases was tested using calcium free buffer (supplemented with 20 mM EDTA) in order to differentiate between the proteolytic enzymes involved in digestion, pro-

Table 1
Effect of the iNOS inhibitor, GED, the peroxynitrite scavenger, FP 15, and the PARS inhibitor, PJ 34, on intestinal polyp number

Treatment	Number of polyps				Total
	Small intestine			Colon	
	Proximal	Middle	Distal		
Control	0	0	0	0	0
Vehicle	6.6 ± 0.6	14.5 ± 1.4	26 ± 2.2	2.3 ± 0.3	49.4 ± 3.3
GED (10 mg/(kg day))	4.9 ± 1	9.3 ± 0.6*	10.3 ± 1*	1.6 ± 0.3	26 ± 2.7 [†]
GED (30 mg/(kg day))	2.9 ± 0.7*	7.1 ± 1*	7.7 ± 0.8*	1 ± 0.3*	18.8 ± 2 [†]
FP 15 (1 mg/(kg day))	5.2 ± 0.7	11.2 ± 1.6	18.8 ± 1.2	1.7 ± 0.2	36.8 ± 2.8 [†]
FP 15 (3 mg/(kg day))	4.6 ± 0.4*	10 ± 1.4*	16.4 ± 1.2*	0.9 ± 0.2*	32 ± 2.6 [†]
PJ 34 (3 mg/(kg day))	8.3 ± 0.9	13.9 ± 1.3	23.9 ± 2.1	1.8 ± 0.1	47.8 ± 3.2
PJ 34 (10 mg/(kg day))	4.1 ± 0.3*	10.3 ± 1.1*	19.3 ± 2*	1 ± 0.3*	34.7 ± 3.4 [†]

Male *Apc*^{min/+} mice were treated from 5 weeks of age with either vehicle (water), GED (10 or 30 mg/(kg day)), FP 15 (1 or 3 mg/(kg day)) or PJ 34 (3 or 10 mg/(kg day)) until 12 weeks of age. Immediately after sacrifice the intestine was removed in its entirety, separated into the various sections and the number of polyps counted in each section by a blinded investigator. Statistical analysis was conducted from *n* = 10–21 per experimental group using one-way ANOVA followed by Student–Newman–Keuls multiple comparisons post-hoc analysis where *P* < 0.05 was considered significant.

* *P* < 0.05 vs. the appropriate vehicle intestine section.

[†] *P* < 0.05 vs. total number of polyps in the vehicle mice.

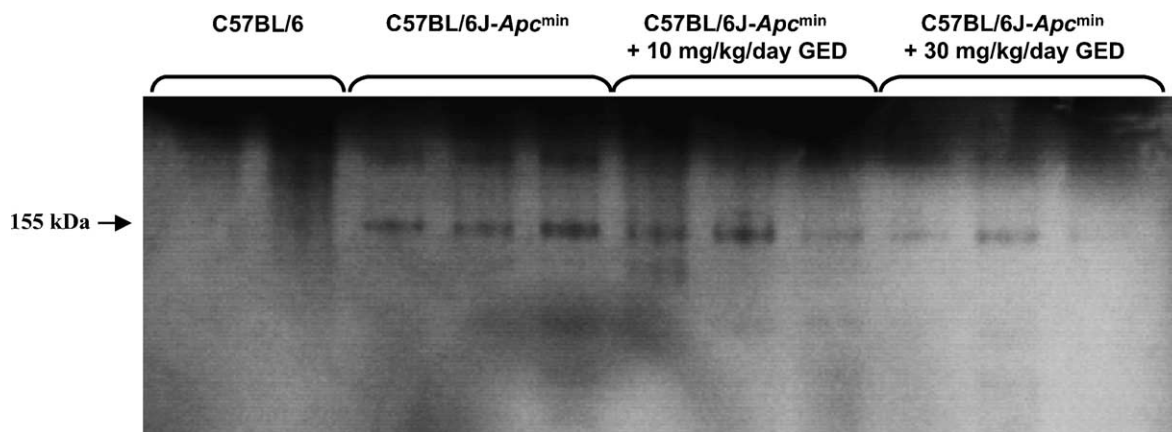


Fig. 1. Activity of 155 kDa metalloproteinase is increased in the intestine of male $Apc^{min/+}$ mice: inhibition by the iNOS inhibitor, guanidinoethylidissulfide. On gelatin zymography gels only one band was clearly detected with a molecular weight of 155 kDa. Activity of this MMP was absent in control mouse intestines but increased in $Apc^{min/+}$ mice with the activity inhibited in mice treated with 30 mg/(kg day) GED. Gel is representative of three gels each with two to three separate mouse extracts for each treatment protocol.

duced in the gut. Only the zymography bands which disappeared in the EDTA buffer showing calcium dependence were considered as MMPs. On the gelatin zymography gels we found several bands which demonstrated calcium dependence (casein zymogra-

phy did not show up calcium dependent bands; results not shown), but only one of these bands which had a molecule weight of 155 kDa was clearly activated in $Apc^{min/+}$ as compared to control mice (Fig. 1). We found no increase in intestinal levels of myeloperoxidase (MPO) indicating there was no neutrophil accumulation in the tissue that may have accounted for the increase in lipid peroxidation (data not shown).

Table 2

Effect of the iNOS inhibitor, GED, the peroxynitrite scavenger, FP 15, and the PARS inhibitor, PJ 34, on intestinal oxidative stress

Treatment	MDA level (nmol/mg protein)
Control mice	0.2 ± 0.02
Vehicle $Apc^{min/+}$ mice	3.3 ± 0.28*
GED (10 mg/(kg day))	1.5 ± 0.1*,†
GED (30 mg/(kg day))	0.9 ± 0.2*,†
FP 15 (1 mg/(kg day))	0.6 ± 0.09*,†
FP 15 (3 mg/(kg day))	0.6 ± 0.06*,†
PJ 34 (3 mg/(kg day))	2.8 ± 0.6*
PJ 34 (10 mg/(kg day))	1.1 ± 0.1*,†

Male $Apc^{min/+}$ mice were treated from 5 weeks of age with either vehicle (water), GED (10 or 30 mg/(kg day)), FP 15 (1 or 3 mg/(kg day)) or PJ 34 (3 or 10 mg/(kg day)) until 12 weeks of age. A biopsy of small intestine was removed and frozen in liquid nitrogen for determination of tissue MDA levels. Statistical analysis was conducted from $n = 10$ –21 mice per experimental group using one-way ANOVA followed by Student–Newman–Keuls multiple comparisons post-hoc analysis where $P < 0.05$ was considered significant.

* $P < 0.05$ vs. control intestine.

† $P < 0.05$ vs. vehicle treated $Apc^{min/+}$ mice.

The iNOS inhibitor, GED, significantly reduced the total number of intestinal polyps at both 10 and 30 mg/(kg day) (Table 1), with a concomitant decrease in tissue MDA level (Table 2). GED treatment also reduced the 155 kDa MMP activity (Fig. 1). Delaying the start of GED treatment from 5 to 8 weeks of age, hence decreasing total treatment time from 7 to 4 weeks, also reduced the number of intestinal polyps (Fig. 2A) and tissue oxidative stress (Fig. 2B) though not as effectively.

The peroxynitrite decomposition catalyst FP 15 at both 1 and 3 mg/(kg day), significantly reduced both the total number of polyps (Table 1) as well as the MDA level (Table 2). Only the highest dose of the PARP inhibitor, PJ 34, 10 mg/(kg day), reduced the total polyp number (Table 1) and the tissue MDA levels (Table 2), while the lower dose tested (3 mg/(kg day)) had no effect on intestinal polyposis or tissue levels of MDA.

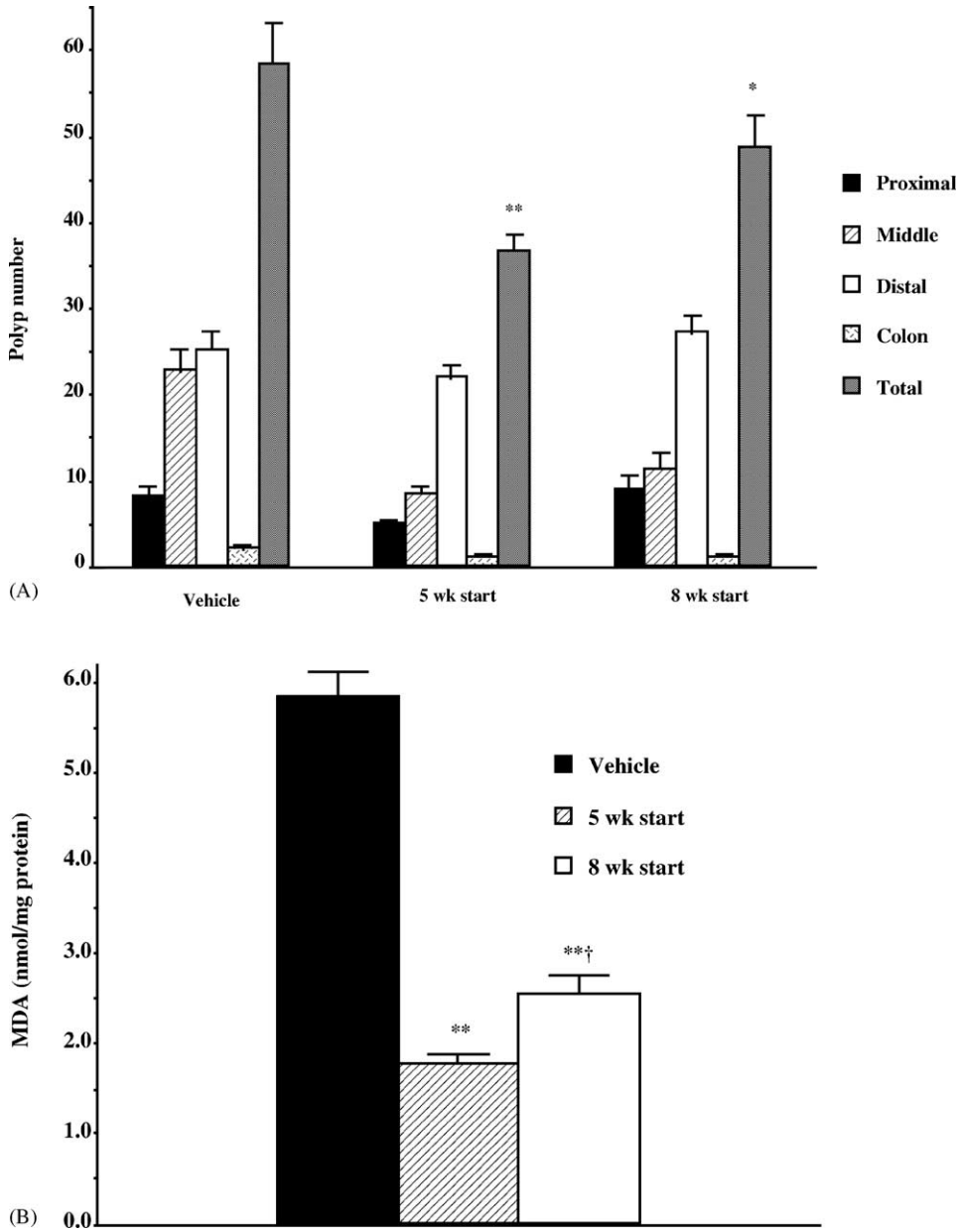


Fig. 2. Effect delaying the start of GED treatment by 3 weeks on intestinal (A) polyp number and (B) oxidative stress. Male *Apc^{min/+}* mice were treated from 5 to 8 weeks of age with either vehicle (water) or GED (30 mg/(kg day)) until 12 weeks of age. Immediately after sacrifice the intestine was removed in its entirety, separated into the various sections and the number of polyps counted in each section by a blinded investigator. A biopsy of small intestine was removed and frozen in liquid nitrogen for determination of tissue MDA levels. Statistical analysis was conducted from $n = 10$ mice per experimental group using one-way ANOVA followed by Student–Newman–Keuls multiple comparisons post-hoc analysis where $P < 0.05$ was considered significant; * $P < 0.05$; ** $P < 0.01$ vs. the intestine from mice where GED treatment started at 5 weeks of age and † $P < 0.05$ vs. the intestine from mice where GED treatment started at 5 weeks of age.

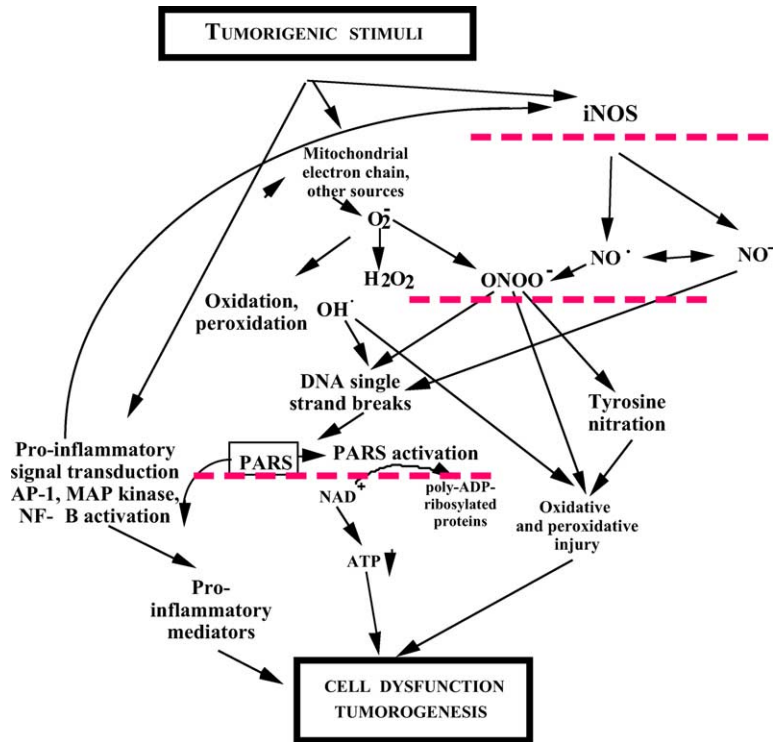


Fig. 3. Proposed scheme depicting the site of action of the iNOS inhibitor, GED (top dashed line), the peroxynitrite scavenger, FP 15 (middle dashed line), and the PARS inhibitor, PJ 34 (bottom dashed line), on polyp generation in the male *Apc^{min/+}* mice. Tumorigenic phenotype is associated with the induction of various cascades culminating in the generation of oxidants and free radicals, including the expression of the inducible isoform of NO synthase (iNOS), which, in turn, produces NO and nitroxyl anion. There is also increased oxidative stress. NO and superoxide reacts to form peroxynitrite. Peroxynitrite, hydroxyl radical and nitroxyl anion induce DNA single strand breakage, as well as increased oxidative stress (including malondialdehyde formation). DNA single strand breakage activates PARS, which, in turn, further enhances the induction of iNOS, can directly trigger necrotic type cell death and may enhance various pro-inflammatory signal transduction pathways.

4. Discussion

We report here a definitive role for nitric oxide and pathways triggered by reactive nitrogen species in intestinal polyposis (Fig. 3). Both a competitive inhibitor of iNOS and a peroxynitrite decomposition catalyst reduced the incidence of intestinal polyps in *Apc^{min/+}* mice. Intestinal polyposis in the *Apc^{min/+}* mice was associated with a dramatic increase in tissue lipid peroxidation, which was reduced by both the specific iNOS inhibitor and a peroxynitrite decomposition catalyst. Interestingly inhibition of PARP also reduced the tumor load of *Apc^{min/+}* mice and the intestinal oxidative stress associated with polyposis. Delaying treatment with the NOS inhibitor GED by 3

weeks was also able to reduce both polyp number and lipid peroxidation. Suppression of the nitric oxide pathway appeared to be more effective than suppression of the poly(ADP-ribose) pathway in reducing tumor load and lipid peroxidation of *Apc^{min/+}* mice. This observation is likely due to the more direct effect of GED and FP 15 on the formation of nitric oxide and related tissue damaging free radicals compared to PJ 34 which functions as an inhibitor of necrosis by preventing catastrophic reductions in cellular ATP levels following extensive DNA damage [33].

We have also demonstrated a marked increase in MDA formation (a classic indicator of oxidative stress in pathophysiological conditions) in the *Apc^{min/+}* mice. To our knowledge, this is the first demonstra-

tion of such an increase in lipid peroxidation in the intestine of the *Apc*^{min/+} mice. Our data on intestinal MPO levels and a more recent comprehensive study by Kettunen et al. [34] has demonstrated that intestinal immunology does not explain tumorigenesis in the *Apc*^{min/+} mice. Our findings demonstrate that the development of polyposis in the *Apc*^{min/+} mice may occur on a strong, hitherto unappreciated basis of marked oxidative/nitrosative stress.

We have also confirmed previous reports of an increase in intestinal MMP activity in *Apc*^{min/+} mice [35], indeed intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin [35]. We have found an increase in activity of a 155 kDa MMP, activity which is reduced by GED treatment implicating nitric oxide and/or oxidative stress as an activation factor for this MMP. Studies using MMP inhibitors have demonstrated a role for these enzymes in tumor progression and growth [36], with inhibitors of MMPs reducing intestinal tumor load in *Apc*^{min/+} mice [37]. It appears MMP activation plays a critical role in intestinal tumorigenesis and that inhibition of MMP activity may account for GEDs protective effect.

The role of iNOS plus nitric oxide and related radical species in intestinal polyposis has proved controversial. Towards the end of 2001 two reports were published describing opposite effects in *Apc*^{min/+} mice with disrupted iNOS gene. Ahn and Ohshima [38] demonstrated a suppression of intestinal polyposis in *Apc*^{min/+};iNOS^{-/-} mice while Scott et al. [39] demonstrated a promotion of intestinal tumorigenesis. Ahn and Ohshima [38] also showed that treatment with aminoguanidine, a moderately selective and moderately potent iNOS inhibitor, or an arginine-free diet also reduced the tumor load of *Apc*^{min/+} mice. The discrepant results of Scott et al. [39] may be due to differences in diet, microflora, infection and environment compared to other laboratories. Indeed the iNOS knockout mouse displays a phenotype consistent with a loss of the cytotoxic actions of nitric oxide but otherwise appears normal with no reports to date of an increased incidence of tumors. However, the majority of published work seems to suggest a pivotal role for nitric oxide in intestinal polyposis, and our data clearly demonstrate a role for reactive nitrogen species. There is also a report showing that iNOS inhibitors can suppress development of azoxymethane-induced aberrant crypt foci in rats [40] and an upregulation of iNOS

protein expression in azoxymethane-induced colonic tumors has been demonstrated, as compared to normal mucosa [41]. Expression of the inducible nitric oxide synthase has been demonstrated in human gastric, colon and breast cancer [42,43]. The inducible isoform of nitric oxide synthase has been shown to be the only isoform involved in stimulating tumor growth, probably through an increase in vascular endothelial growth factor production [44]. Interestingly, a study by Konopka et al. implicated peroxynitrite over nitric oxide as the relevant species which increases VEGF mRNA expression and release [44].

Inhibiting the nuclear enzyme PARP (which has historically been viewed primarily as a DNA repair enzyme) and reducing tumor formation appears at first sight to be a contradiction. One of the first responses of a cell to DNA single strand breakage is the synthesis of poly(ADP-ribose) by PARP. It has also been suggested that PARP deficiency causes genomic instability via shortening telomeres whose dysregulation is proposed to be a critical event in the development of cancers. There have been reports of PARP inhibitors enhancing and accelerating cancer development in animal models of liver carcinogenesis [45], skin tumors [46], and pancreatic β -cell tumors [47], all suggesting that inhibition of PARP activity promotes tumorigenesis. However, the PARP knockout mouse develops normally and is fertile and despite reports of genomic instability these mice have not been reported to be prone to tumor development [48]. Therefore, our data stand in apparent conflict with what has been published in previous reports. We have found that a specific PARP inhibitor, PJ 34, reduced intestinal polyposis in *Apc*^{min/+} mice and reduced intestinal oxidative stress. We believe that the latter point is likely to be the key to the mechanism of action. In agreement with our observations, Conde et al. [49] reported that loss of PARP-1 increases tumor latency in p53-deficient mice. Mice lacking p53 are predisposed to developing tumors due to defective cell cycle checkpoints, resistance to DNA-damage induced apoptosis and upregulation of iNOS resulting in chronic oxidative stress [49]. Indeed nitric oxide involvement in the spontaneous tumorigenesis in the p53-deficient mouse has been demonstrated to be pivotal with previous work demonstrating that calorie restriction [50,51] or administration of the steroids dehydroepiandro-

terone [52] and 16 α -fluro-5-androsten-17-one [53] reduce spontaneous tumorigenesis by a mechanism of inhibition of iNOS expression [54]. When in p53 knockout mice the PARP-1 gene was disrupted, the tumor free survival increased by 50% compared to the p53 knockout with wild type PARP mice [49]. The increased survival was associated with a decrease in iNOS expression and nitric oxide level in these mice [49]. Therefore, it appears that PARP-1's role in tumorigenesis is linked to the cells' oxidative status. PARP has a functional link to activation of the transcriptional factor NF- κ B and through this pathway PARP inhibitors can reduce the cellular expression of iNOS in response to various stimuli [55] (Fig. 3). PARP inhibitors have been suggested as possible agents to sensitize cancer cells to chemotherapy [56]. Data presented here and published by other groups suggest that PARP inhibitors may also have additional beneficial effects in reducing tumorigenesis in situations where the development of tumors occurs on the basis of chronic pro-inflammatory/pro-oxidant stimuli.

In conclusion, the current report identifies iNOS, peroxynitrite and PARP as downstream effectors of intestinal polyp formation in the *Apc*^{min/+} mice. One can hypothesize that targeting the generation of reactive nitrogen species and oxidative stress may be a possible therapeutic approach to cancer prevention or treatment.

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