Induction of NAD(P)H Quinone:Oxidoreductase1 Inhibits Carcinogen-induced Aberrant Crypt Foci in Colons of Sprague-Dawley Rats

Asher Begleiter, Kosala Sivananthan, Thomas J. Curphey, and Ranjana P. Bird

Manitoba Institute of Cell Biology, CancerCare Manitoba, Winnipeg, Manitoba R3E 0V9 Canada [A. B., K. S.]; Departments of Internal Medicine [A. B., K. S.], Pharmacology and Therapeutics [A. B.], and Human Nutritional Sciences [R. P. B.], University of Manitoba, Winnipeg, Manitoba R3E 0V9 Canada; and Department of Pathology, Dartmouth College, Hanover, New Hampshire 03755 [T. J. C.]

Abstract
Phase II detoxifying enzymes like NAD(P)H (quinone acceptor)oxidoreductase1 (NQO1), glutathione S-transferases (GST), and UDP-glucuronosyltransferases (UGT) may play an important role in preventing carcinogen-induced cancers. Inducers of these enzymes have been shown to inhibit carcinogen-induced colon tumors in rat and mouse models. However, it has not been clearly demonstrated that NQO1 contributes to this effect. We examined the effect of NQO1 inducers on colon carcinogenesis using an aberrant crypt foci (ACF) rat model. Sprague-Dawley rats were fed control diet or diet containing 400 ppm dimethyl fumarate or 200 ppm oltipraz for 7 days, and Phase II enzymes in rat colon and liver were measured. Dimethyl fumarate significantly increased NQO1 and GST activities in colon and liver but did not increase UGT activities in these tissues. In contrast, oltipraz significantly increased NQO1 activities in colon and liver and produced a small increase in GST activity in the liver but did not increase GST activity in the colon or UGT activities in the liver or colon. Sprague Dawley rats were fed control diet or diet containing 200 ppm oltipraz and then treated with the carcinogens azoxymethane or methyl nitrosourea. Both carcinogens produced ACF in all of the rat colons, but rats fed oltipraz diet had significantly fewer ACF than those fed control diet. This protective effect was reversed in rats treated with the NQO1 inhibitor, dicoumarol. However, treatment with oltipraz did not alter the distribution of crypt multiplicities in the ACF. These studies demonstrated that induction of NQO1 plays a significant role in inhibiting initiation of carcinogen-induced ACF in Sprague-Dawley rats. This provides the first direct evidence that NQO1 may play a role in preventing colon cancer. The study also found that oltipraz added to the diet of Sprague-Dawley rats selectively increased NQO1 activity in colon mucosa with no increase in GST and UGT activities in these tissues. Thus, this model will be useful for further investigating the role of NQO1 in prevention of colon cancer.

Introduction
Colorectal cancer is a common malignancy representing 11% of new cancer cases and 10% of cancer deaths in North America (1). The majority of these cancers are of a sporadic nature with <5% being caused by inherited colon cancer syndromes. Most colon cancers develop from epithelial cells of the colon and are initiated by exposure to carcinogens. Initiated cells may then progress through a series of well-defined precancerous lesions and premalignant and malignant stages.

There is considerable evidence that dietary factors can influence the risk of colon tumors. Animal fat is positively related to colon cancer incidence, whereas fruit and vegetable intake is negatively related (2). Up to 90% of colon cancers may be preventable by dietary changes (3). It is generally believed that diet can influence the risk of colon tumors because components in diet can act as carcinogens that initiate carcinogenesis or promoters that influence tumor progression. Alternatively, dietary components may prevent the initiation of the carcinogenesis process by helping to inactivate or remove the carcinogens or may inhibit progression of carcinogenesis by detoxifying promoters or directly inhibiting the evolution of the initiated cells to cancer cells.

Considerable effort has been directed toward the use of dietary and related agents as a way of reducing cancer risk (4). Agents like isothiocyanates and dithiolethiones, which occur naturally in cruciferous vegetables, and their analogues are under investigation as chemopreventive agents (5–7). The presence of these agents in the human diet and their low toxicity make them attractive options in cancer prevention strategies (8). Isothiocyanates and dithiolethiones are among the most potent known chemopreventive agents present in food (6). It has been proposed that these agents exert their chemoprotective effects by inducing Phase II detoxifying enzymes like NQO1,3 GSTs, and UGT (5–8). Phase II enzyme induction results in modification and rapid excretion of carcinogens, producing a chemoprotective effect, e.g., Sugie et al. (9) reported a lower colonic tumor incidence in animals fed benzyl isothiocyanate before carcinogen exposure. However, there is also evidence that these agents may work at the post-initiation stage of carcinogenesis and that these agents may directly induce apoptosis in cells (10). Smith et al. (10) observed lower ACF formation...

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1 The abbreviations used are: NQO1, NAD(P)H (quinone acceptor)oxidoreductase1; GST, glutathione S-transferase; MNU, N-methyl-nitrosourea; AOM, azoxymethane; ACF, aberrant crypt foci; UGT, UDP-glucuronosyltransferase.

2 To whom requests for reprints should be addressed, at Manitoba Institute of Cell Biology, 675 McDermot Avenue, Winnipeg, MB R3E 0V9, Canada. Phone: (204) 787-2155; Fax: (204) 787-2190; E-mail: begleit@cc.umanitoba.ca.

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in animals fed allyl isothiocyanate after carcinogen exposure. Thus, the exact mechanisms involved in the chemopreventive effects of isothiocyanates and dithiolethiones, and the importance of specific Phase II enzymes, are still unknown.

Oltipraz is a dithiolethione analogue that was originally used as an antiparasitic agent in humans (11). It has been shown to protect against chemically induced carcinogenesis in the lung, foreestomach, trachea, colon, breast, skin, and urinary bladder in a variety of rodent models (7, 12). Oltipraz has also been studied as a cancer chemopreventive agent in clinical trials (13–15).

Preliminary results from these trials indicate that it can be safely administered to humans and that it reduces activation and increases excretion of the carcinogen aflatoxin M1. Specifically for colon cancer, oltipraz increased the levels of NQO1, GST, and UGT in the livers and colons of F344 rats and reduced the incidence of AOM-induced colon carcinomas in these rats when given either before, or after, the carcinogen (16). However, because all three Phase II enzymes were induced by oltipraz in this rat model, it is not clear whether induction of NQO1 contributed to the inhibitory effect.

NQO1 is a flavoprotein that catalyzes two electron reduction of quinones and nitrogen oxides (17, 18). Although the major function of this protein may be to reduce the formation of reactive oxygen species by decreasing one electron reduction and the associated redox cycling (19), it also plays a role in activation of some anticancer drugs (20) and cancer prevention (17, 18, 21). Recent studies suggest that NQO1 may also be involved in regulation of the transcription factor p53 and apoptosis (22, 23).

Several human oxidoreductase genes have been identified (24, 25), but NQO1 appears to be the most important for prevention of carcinogen-induced tumors (18). The NQO1 gene, located on chromosome 16q2.2, is 20 kb in length and has six exons and five introns (24). The protein consists of two identical protein subunits of Mr ~30,000 whose expression is transcriptionally controlled (17). The enzyme is found mainly in cytosol but is also present in mitochondria, microsomes, Golgi (17), and the nucleus (26). It is ubiquitous in eukaryotes, but levels vary in different tissues (17, 27). A polymorphism involving a base change from cytosine to thymine at base 609 of the NQO1 gene occurs at high incidence in the human population (28–30).

The most abundant form of the NQO1 protein appears to have little or no enzyme activity (28, 31), and cells with one G→T allele may have decreased levels of enzyme activity, but cells with two G→T alleles have no activity (28, 32). However, the specific role of NQO1 in colon carcinogenesis and cancer prevention is not understood.

ACF occur in carcinogen-treated rodent colons and have been suggested to be preneoplastic lesions (33). Aberrant crypts can be identified by their increased size, irregular and dilated luminal opening, thicker epithelial lining, and pericytoplatic zone (34). Colon carcinogens like AOM and intrarectally administered MNU induce ACF in a dose-related manner, and known inhibitors or promoters of colon carcinogenesis inhibit or promote the number and growth of ACF. Similarities have been noted between the lesions found in rat and human colons. Although only a small proportion of ACF go on to become colon tumors, the number and multiplicity of these lesions are predictive of tumor incidence (33). Because ACF occur at an early stage of carcinogenesis, the use of these lesions as a biological end point significantly reduces the time required to study the effects of carcinogens and modifying agents (33–36) and the mechanisms involved (36).

Although many carcinogens induce ACF or colon tumors in rats, AOM administered s.c. or intrarectal MNU have been most widely used in this model (34). AOM requires activation in the liver (37) and produces a tumor colonies. AOM is normally cleared from the animal in <24 h. In contrast, MNU is a direct acting carcinogen that does not require activation and generally produces tumors in most locations where it is applied (38, 39). MNU is absorbed over a 2–3-h period after an intragastric dose, but because it is a highly reactive alkylating agent, 40% remains in the body bound to macromolecules (40).

In this study, we investigated induction of Phase II enzymes in Sprague-Dawley rats by oltipraz and another Phase II enzyme inducer, dimethyl fumarate, and examined the effect of oltipraz on carcinogen-induced ACF formation. We used dicoumarol, an inhibitor of NQO1, to specifically determine whether NQO1 contributes to inhibition of colon carcinogenesis.

Materials and Methods

Sprague-Dawley 5–6-week male rats were purchased from the University of Manitoba Central Animal Care Breeding Facility, Winnipeg, Canada. Modified AIN-76A powdered diet containing 13% dextrose and 52% corn starch was obtained from Harlan Teklad (Madison, WI). Oltipraz was prepared as we have described previously (41). Oltipraz or dimethyl fumarate was mixed into the modified AIN-76A diet when required. AOM, MNU, dicoumarol, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide were obtained from Sigma (St. Louis, MO). Dimethyl fumarate was purchased from Aldrich (Milwaukee, WI).

For studies of induction of Phase II enzymes, Sprague-Dawley rats were fed control diet or diet containing 200 ppm oltipraz or 400 ppm dimethyl fumarate for 7 days. Rats were euthanized, colon mucosa and liver were excised, and NQO1, GST, and UGT (42–44) activities were measured as has been described previously. Enzyme activities in colon mucosa and liver of control fed and inducer fed animals were compared by t tests.

For studies of the effect of oltipraz on AOM-induced ACF, Sprague-Dawley rats were fed control diet or diet containing 200 ppm oltipraz from day 0 to 15. On days 7 and 14, rats received AOM at 15 mg/kg by s.c. injection. The rats in each dietary group were also treated with dicoumarol at 15 mg/kg or saline by tail-vein injection 2 h before and 6 h after AOM. All of the rats were then fed control diet for 4 weeks and euthanized. Colons were removed, fixed in 10% buffered formalin, and stained with methylene blue, and ACF number and multiplicity were enumerated as we have described previously (33, 34). ACF numbers in the four treatment groups were compared by one-way ANOVA (Student-Newman-Keuls Method). The distribution of crypt multiplicities in the four groups was analyzed by repeated measures ANOVA.

For studies of the effect of oltipraz on MNU-induced ACF, Sprague-Dawley rats were fed control diet or diet containing 200 ppm oltipraz from day 0 to 20. On days 7, 10, 12, 14, 17, and 19, rats received MNU at 6.5 mg/kg by intrarectal injection. The rats in each dietary group were also treated with dicoumarol at 15 mg/kg or saline by tail-vein injection 2 h before each MNU injection. All of the rats were then fed control diet for 6 weeks and euthanized. Colons were removed, fixed in 10% buffered formalin, and stained with methylene blue, and ACF number and multiplicity were enumerated (33, 34). ACF numbers in the four treatment groups were compared by one-way ANOVA (Student-Newman-Keuls Method). The distribution of crypt multiplicities in the four groups was analyzed by repeated measures ANOVA.
Results

Effect of Dimethyl Fumarate and Oltipraz on Phase II Detoxifying Enzymes. To determine the effect of dimethyl fumarate and oltipraz on Phase II enzyme activities in rats, Sprague-Dawley rats were fed control diet or diet containing 400 ppm dimethyl fumarate or 200 ppm oltipraz for 7 days. Rat colon mucosa and livers were excised, and NQO1, GST, and UGT activities were measured. Dimethyl fumarate increased NQO1 in colon mucosa by 7-fold ($P < 0.001$) and in liver by 6-fold ($P < 0.001$; Fig. 1). Dimethyl fumarate did not significantly increase UGT activities in colon mucosa or liver. Oltipraz increased NQO1 activity in colon mucosa by 2.5-fold ($P < 0.001$) and in liver by 1.6-fold ($P < 0.001$; Fig. 2). Oltipraz did not increase UGT activities in colon mucosa or liver or GST activity in colon mucosa but did produce a small increase of 1.2-fold ($P < 0.05$) in GST activity in liver.

Effect of Oltipraz on Carcinogen-induced ACF. To determine the effect of oltipraz on induction of ACF by AOM, Sprague-Dawley rats were fed control diet or control diet containing 200 ppm oltipraz from day 0 to 15. On days 7 and 14, the rats received AOM at 15 mg/kg and were also treated with dicoumarol at 15 mg/kg or saline 2 h before and 6 h after each
AOM treatment. All of the rats were then fed a control diet for 4 weeks. Colons were removed, and ACF number and multiplicity were enumerated. Rats fed a control diet that received AOM and were treated with saline (control) had \( 180 \pm 14 \frac{ACF}{rat} \) (\( n = 20 \)), whereas rats fed the oltipraz diet that received AOM and were treated with saline had \( 119 \pm 11 \frac{ACF}{rat} \) (\( n = 20 \)), and this difference was statistically significant (\( P < 0.05 \); Fig. 3). Oltipraz fed rats that received AOM and were treated with dicoumarol had \( 206 \pm 16 \frac{ACF}{rat} \) (\( n = 19 \)), and this was statistically different from the oltipraz fed rats treated with saline (\( P < 0.05 \)). The number of ACF in rats fed control diet and treated with dicoumarol was not statistically different from control rats. In addition, oltipraz did not significantly alter the distribution of crypt multiplicities of the ACF (Table 1).

To determine the effect of oltipraz on induction of ACF by MNU, Sprague-Dawley rats were fed control diet or diet containing 200 ppm oltipraz from day 0 to 20. On days 7, 10, 12, 14, 17, and 19, the rats received MNU at 6.5 mg/kg by intrarectal injection. The rats in each dietary group were also treated with dicoumarol at 15 mg/kg or saline by tail-vein injection 2 h before each MNU injection. All of the rats were then fed control diet for 6 weeks and euthanized. Colons were removed, and ACF number and multiplicities were enumerated. ACF numbers in the four treatment groups were compared by one-way ANOVA (Student-Newman-Keuls Method). Results are the mean ± SE of 18–20 rats.

![Fig. 3](image-url). Effect of dietary oltipraz on AOM-induced ACF formation. Sprague-Dawley rats were fed control diet or diet containing 200 ppm oltipraz from day 0 to 15. On days 7 and 14, rats received AOM at 15 mg/kg by s.c. injection. The rats in each dietary group were also treated with dicoumarol at 15 mg/kg or saline by tail-vein injection 2 h before and 6 h after AOM. All of the rats were then fed control diet for 4 weeks and euthanized. Colons were removed, and ACF number and multiplicities were enumerated. ACF numbers in the four treatment groups were compared by one-way ANOVA (Student-Newman-Keuls Method). Results are the mean ± SE of 19–20 rats.

![Table 1](image-url). Effect of oltipraz on crypt multiplicity of AOM-induced ACF

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![Fig. 4](image-url). Effect of dietary oltipraz on MNU-induced ACF formation. Sprague-Dawley rats were fed control diet or diet containing 200 ppm oltipraz from day 0 to 20. On days 7, 10, 12, 14, 17, and 19, rats received MNU at 6.5 mg/kg by intrarectal injection. The rats in each dietary group were also treated with dicoumarol at 15 mg/kg or saline by tail-vein injection 2 h before each MNU injection. All of the rats were then fed control diet for 6 weeks and euthanized. Colons were removed, and ACF number and multiplicities were enumerated. ACF numbers in the four treatment groups were compared by one-way ANOVA (Student-Newman-Keuls Method). Results are the mean ± SE of 18–20 rats.

and this difference was statistically significant (\( P < 0.05 \); Fig. 4). Oltipraz fed rats that received MNU and were treated with dicoumarol had \( 28 \pm 3 \frac{ACF}{rat} \) (\( n = 18 \)), and this was statistically different from the oltipraz fed rats treated with saline (\( P < 0.05 \)). The number of ACF in rats fed control diet and treated with dicoumarol was not statistically different from control rats. Oltipraz did not significantly alter the distribution of crypt multiplicities of the ACF (Table 2).

**Discussion**

Phase II detoxifying enzymes like NQO1, GST, and UGT are thought to play an important role in preventing carcinogen-induced colon cancers (6, 8). NQO1 is a reductive enzyme that...
NQO1 Inhibits Carcinogen-induced Aberrant Crypt Foci

enzymes previously (16). Feeding oltipraz for was not toxic to the rats and had been shown to induce Phase II enzymes in these effects, are still unknown. The exact mechanisms involved in the chemopreventive effects of Phase II detoxifying enzymes, isothiocyanate, when this agent was fed before carcinogen exposure. However, there is also evidence that these agents may work at the post-initiation stage of carcinogenesis and that these agents may directly induce apoptosis in cells. Smith et al. (10) found lower ACF formation in animals fed another Phase II enzyme inducer, allyl isothiocyanate, after carcinogen exposure. Similarly, oltipraz (11) has been shown to protect against chemically induced carcinogenesis in the colon, lung, fore-stomach, trachea, breast, skin, and urinary bladder in a variety of rodent models (7) and has been studied as a cancer chemopreventive agent in clinical trials (13–15). Specifically for colon cancer, oltipraz increased the levels of NQO1, GST, and UGT in the livers and colons of F344 rats and reduced the incidence of AOM-induced colon carcinomas in these rats when given either before or after the carcinogen (16). Thus, the exact mechanisms involved in the chemopreventive effects of Phase II enzyme inducers, and the importance of specific Phase II enzymes in these effects, are still unknown.

In this study, we investigated induction of Phase II enzymes in Sprague-Dawley rats by dimethyl fumarate and oltipraz and examined the effect of oltipraz on carcinogen-induced ACF formation. ACF are preneoplastic lesions (33) that occur during the distribution of ACF multiplicities, providing further evidence that the effects of NQO1 in this study were on the prevention of carcinogen-induced colon carcinogenesis. However, it is likely that other Phase II detoxifying enzymes like GST and UGT are also important in colon cancer prevention and that a greater inhibitory effect would be observed if all of these enzymes were induced coordinately.

In these studies, rats were treated with dicoumarol 2 h before AOM injection and 6 h after AOM to confirm that the effects of oltipraz were caused by induction of NQO1. Dicoumarol is a competitive inhibitor of NQO1 with a half-life of ~7.5–9 h in rats (49). Rats were treated with dicoumarol at these times because AOM remains present in rats for 12–24 h, thus ensuring that the NQO1 was inhibited during the time when AOM was present. In this study, we did observe a small increase in ACF number in rats fed oltipraz diet and treated with dicoumarol compared with control rats. However, this increase was only 14% and likely has little biological significance, because dicoumarol alone did not significantly affect ACF numbers, and no similar increase was seen in oltipraz- and dicoumarol-treated rats when MNU was used as the carcinogen.

Because AOM is a pro-carcinogen that must be activated in the liver, and oltipraz induced NQO1 in both liver and colon mucosa, it was not clear where the enzyme was producing its inhibitory effect. Thus, we carried out a similar study with the direct acting carcinogen, MNU, which does not require activation and produces its carcinogenic effect directly in the colon when given intrarectally. Using the oltipraz fed University of

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reduces quinone and nitrogen oxide functional groups (17, 19), whereas GST and UGT help conjugate carcinogens and other xenobiotics to glutathione and glucuronic acid, respectively (6, 8). Reduction by NQO1 may directly inactivate carcinogens and/or make them better substrates for GST and UGT, making it easier to remove them from cells (21). A variety of agents, including dietary components, can induce Phase II detoxifying agents (45–47) and have been shown to inhibit colon carcinogenesis. Sugie et al. (9) obtained a lower colonic tumor incidence in animals fed the Phase II enzyme inducer, benzyll isothiocyanate, when this agent was fed before carcinogen exposure. However, there is also evidence that these agents may work at the post-initiation stage of carcinogenesis and that these agents may directly induce apoptosis in cells. Smith et al. (10) found lower ACF formation in animals fed another Phase II enzyme inducer, allyl isothiocyanate, after carcinogen exposure. Similarly, oltipraz (11) has been shown to protect against chemically induced carcinogenesis in the colon, lung, fore-stomach, trachea, breast, skin, and urinary bladder in a variety of rodent models (7) and has been studied as a cancer chemopreventive agent in clinical trials (13–15). Specifically for colon cancer, oltipraz increased the levels of NQO1, GST, and UGT in the livers and colons of F344 rats and reduced the incidence of AOM-induced colon carcinomas in these rats when given either before or after the carcinogen (16). Thus, the exact mechanisms involved in the chemopreventive effects of Phase II enzyme inducers, and the importance of specific Phase II enzymes in these effects, are still unknown.

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Manitoba Sprague-Dawley rat model, we studied the effect of NQO1 induction on MNU-induced ACF formation. Rats fed a diet containing 200 ppm oltipraz before, and during, treatment with the carcinogen MNU had 72% fewer ACF than rats fed a control diet, and this result was statistically significant ($P < 0.05$). Dicoumarol treatment significantly reduced the inhibitory effect of oltipraz on ACF formation ($P < 0.05$). However, in this case, dicoumarol did not completely reverse the inhibitory effect of oltipraz on ACF formation. This may have been because the rats were given only a single treatment of dicoumarol. This was done because MNU is a highly reactive carcinogen, and we believed that a single dicoumarol treatment would be sufficient to inhibit NQO1 during the time when the MNU was present. We again found that oltipraz, with and without dicoumarol, did not significantly alter the distribution of ACF multiplicities, providing further evidence that the effects of NQO1 in this study were on the initiation of carcinogenesis (Table 2).

This result confirmed our previous findings with AOM and also provided evidence that oltipraz was producing its inhibitory effect in the colon. Because oltipraz induced NQO1, but not GST and UGT, in the colon of this Sprague-Dawley rat model, this study provided further evidence that induction of NQO1 alone was sufficient to produce initiation inhibition of colon carcinogenesis. It is not known at this time how NQO1 produces its inhibitory effect on initiation of colon carcinogenesis. AOM and MNU are thought to produce their carcinogenic effects by forming DNA adducts. As both AOM and MNU contain nitrogen oxide groups, they are likely substrates for NQO1. Reduction of the nitrogen oxide group by NQO1 may inactivate the carcinogens directly and/or may increase their conjugation to glutathione or glucuronic acid and cellular excretion. Inactivation of NQO1 by dicoumarol would prevent these effects.

In summary, these studies demonstrated that induction of NQO1 alone can inhibit initiation of carcinogen-induced ACF in rats. This provides the first direct evidence that NQO1 may play a role in prevention of colon cancer. This finding may have significance in human colon cancer, as 20–60% of the human population has reduced or no NQO1 activity because of the high incidence of the base 609 polymorphism in the NQO1 gene (30). The study also found that oltipraz added to the diet of University of Manitoba bred Sprague-Dawley rats increased NQO1 activities in colon mucosa with no increase in the activities of GST and UGT in these tissues. Thus, this model will be useful for further investigating the role of NQO1 in prevention of colon cancer.

Acknowledgments

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References


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