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Advances in Brief

The Chemopreventive Agent Oltipraz Stimulates Repair of Damaged DNA[1]

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Abstract

Carcinogens may damage DNA either through the production of radicals that cause base modification *in situ* or through the formation of bulky adducts at relatively nucleophilic sites. Preclinical studies have demonstrated that administration of the dithiolethione oltipraz protects laboratory animals from the development of tumors following subsequent exposure to a variety of carcinogens. This may occur through a mechanism involving the induction of detoxicating gene expression. In some models, oltipraz treatment following carcinogen exposure may also confer protection. To investigate a possible mechanism for this observation, we studied the effects of oltipraz on base excision repair and platinum-DNA damage formation and removal. No effect of oltipraz was observed on base excision repair as determined by an *in vitro* assay measuring the repair of apurinic/apyrimidinic sites by untreated and oltipraz-treated HT-29 whole-cell extracts. Treatment of HT-29 cells with cisplatin in the absence or presence of 30 and 100 μM oltipraz decreased the accumulation of platinum in DNA. A dose-dependent reduction in DNA platination was also observed in purified DNA treated concurrently with cisplatin and increasing concentrations of oltipraz. When DNA was first platinated and subsequently incubated with oltipraz, no decrease in platinum content in DNA was found. Preincubation of HT-29 cells with oltipraz enhanced the rate of removal of total platinum-DNA adducts and interstrand cross-links. These data support a novel mechanism through which dithiolethiones may protect carcinogen-exposed animals from tumor formation and may expand their potential role in the clinic.

Introduction

Epidemiological studies demonstrate that the risk of developing certain tumors, especially colon cancer, may be diminished by a diet rich in cruciferous vegetables (1–3). Such vegetables (including broccoli, cabbage, and cauliflower) are a rich source of dithiolethiones, which are believed to contribute to their protective effects (4). Support for this concept derives from the fact that a synthetic dithiolethione, oltipraz, has been shown to protect laboratory animals from developing tumors following exposure to several DNA-damaging carcinogens (5). The protective effect is believed to result from elevation of the activity of one or more detoxicating enzymes through an inductive effect on gene transcription (6, 7). In recent clinical trials, oltipraz has been shown to induce the transcription of detoxicating genes in human tissues (8). Based on these data, the potential of oltipraz to protect patients at high risk for colorectal cancer from the development of this disease is being investigated.

The initial rodent studies with oltipraz established that the addition of the drug to the laboratory diet yielded a dose-dependent decrease in the number of tumors that resulted from a subsequent exposure to carcinogens, including diethylnitrosamine, uracil mustard, benzo(*a*)pyrene, azoxymethane, aflatoxin B_1, and *N*-nitrobis-(2-oxypropyl)-amine hydroxybutylnitrosamine (5, 9–12). It was also shown that although pretreatment of the animals with oltipraz was protective, so was the administration of oltipraz following carcinogen exposure (13). This latter observation suggested an effect of oltipraz even after DNA damage by the carcinogen. Because such a mechanism would greatly broaden the potential value of a protective drug of this nature, we examined the effect of oltipraz on DNA repair.

Two forms of DNA repair were studied. Base excision repair was measured using whole-cell extracts from oltipraz-treated cells in assays of the repair of AP[3] sites. NER was measured using the anticancer drug cisplatin as a model. It has been shown previously that platinum-DNA adducts are repaired predominantly by NER (14). The results indicate that NER but not base excision repair is stimulated by oltipraz in a dose-dependent fashion. These results expand the potential role of drugs of this nature to applications broader than cancer prevention and justify prevention studies in larger populations.

Materials and Methods

Cells and Reagents. Human HT29 cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in DMEM medium containing 10% fetal bovine serum. Cisplatin was obtained from Bristol-Myers Squibb (Syracuse, NY). Oltipraz was obtained from the National Cancer Institute (Bethesda, MD).

Base Excision Repair. The covalently closed circular DNA carrying either a natural AP site or a 3-hydroxy-2-hydroxymethyltetrahydrofuran (tetrahydrofuran) residue (a synthetic analogue of the AP site) with a ^{32}P at the 5′ side of the lesion was prepared as described previously (15). The whole-cell extracts were prepared as described by Tanaka *et al.* (16). The protein concentrations were determined by the Bradford assay (Bio-Rad, Richmond, CA).

Repair reactions containing 10 μg of protein from the whole-cell extract were carried out as described previously (15). After recovery from the repair reaction, the DNA samples were digested with *Pvu*II and *Xenopus laevis* AP endonuclease and analyzed by gel electrophoresis in a 6% polyacrylamide gel with 8 M urea. The gel was dried and subjected to autoradiography with an X-ray film or scanned with a Fuji BAS1000 imaging system for quantitative analysis.

Platinum-DNA Adduct Formation and Removal in HT-29 Cells. The formation and removal of total platinum-DNA adducts was studied by incubating triplicate flasks of cells (1×10^8) for 24 h with oltipraz 0, 30, or 100 μM, followed by incubation with cisplatin concentrations of 0, 10, 20, 30, 40, and 50 μM for 4 h. DNA was isolated by the phenol/$CHCl_3$ method and processed for platinum determination by AAS. DNA platination was measured using a Perkin-Elmer model 3100 atomic absorption spectrometer equipped with a graphite furnace as described previously (17). For repair experiments, HT-29 cells were pretreated with 0, 30, or 100 μM of oltipraz for 24 h followed by a 4-h incubation with the concentration of cisplatin required to obtain similar initial levels of total platinum-DNA adducts. Cells were either harvested immediately or fresh medium was added, and the cells were incubated for various repair times. DNA was isolated, restriction-digested with *Hin*dIII,

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[3] The abbreviations used are: AP, apurinic/apyrimidinic; NER, nucleotide excision repair; AAS, atomic absorption spectrometry.

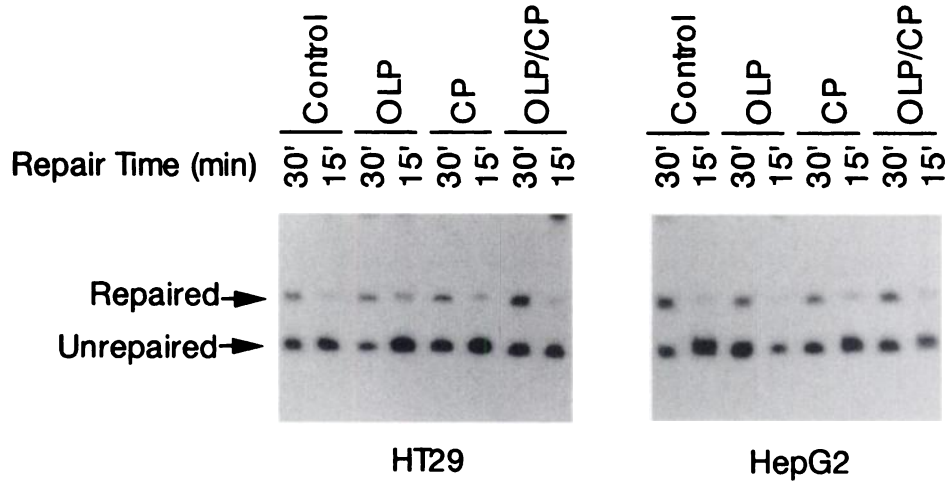

Fig. 1. Effect of oltipraz (*OLP*; 100 μM), cisplatin (*CP*; 25 μM), and combined oltipraz and cisplatin treatment of HT29 colon adenocarcinoma and HepG2 hepatoma cells on base excision repair. Cells were pretreated for 24 h (*OLP*), or 4 h (*CP*), or both, or neither (control). Whole-cell extracts were prepared and incubated with a plasmid containing a natural AP site as described in "Materials and Methods." The repaired and unrepaired plasmids were separated by electrophoresis, and relative repair was quantitated as a percentage of the total signal. No differences were observed among the treatment conditions.

and either processed for total platinum content by AAS or subjected to renaturating agarose gel electrophoresis for cisplatin interstrand cross-link determination (18). Briefly, 2 μg of DNA were incubated at 60°C-65°C for 5 min in 0.2 N NaOH and immediately placed on ice. Samples were loaded onto a 0.5% agarose gel prepared in 40 μM Tris-acetate buffer containing 1 μM EDTA and electrophoresed for 4 h at 100 V. Following Southern blotting, membranes were hybridized with the ABE_{II} probe, which recognizes a 17-kb *Hin*dIII fragment of the 28S rRNA gene. Membranes were washed and subjected to autoradiography. Histograms were generated for each lane using a Fuji BAS1000 imaging system, and the fraction of cross-linked strands was determined by weight analysis of the peaks. The average number of interstrand cross-links per fragment was calculated using the Poisson distribution equation, $\ln(1 - Fc)$, where Fc is the fraction of DNA strands containing cross-links.

Platinum-DNA Adduct Formation and Removal in Purified DNA. The formation of platinum-DNA adducts in purified DNA was examined by incubating 50 μg of DNA purified from untreated HT-29 cells with 10 ng of cisplatin and either 0, 30, or 100 μM of oltipraz in 450 μl of TE buffer (10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA) for 4 h at 37°C. For platinum-removal studies, DNA isolated from HT-29 cells that were treated with 100 μM cisplatin for 4 h was subsequently incubated in the presence of 0, 30, or 100 μM of oltipraz in 450 μl of TE buffer for 0, 2, or 4 h at 37°C. The reactions were extracted in phenol/$CHCl_3$ and precipitated in ethanol; then the platinum content was determined by AAS.

Results and Discussion

Consistent with studies in rodent model systems, we have shown previously that cultured HT29 colon adenocarcinoma cells respond to oltipraz treatment by up-regulating the expression of genes involved in detoxication, including DT-diaphorase and γ-glutamylcysteine synthetase (19).[4] Maximal induction is observed at 100 μM exposure for 24 h, whereas concentrations less than 30 μM have little effect. This up-regulation is also associated with increased expression of the ubiquitous transcription factors nuclear factor-κB and AP-1 (19).[5] Therefore, these cells respond to oltipraz treatment in a manner similar to that of the tissues of animals treated with oltipraz, in whom a chemoprotective effect is observed (7).

Carcinogens may damage DNA either through the production of radicals that cause base modification *in situ* or through the formation of bulky adducts at relatively nucleophilic sites (such as the N7 of guanine; Ref. 20). The modified bases that result from radical formation are generally thought to be repaired by base-excision repair (21). In vertebrates, the reaction of base-excision repair can proceed in one of two alternative pathways: the DNA polymerase β-dependent pathway; and the proliferating cell nuclear antigen-dependent pathway (15, 22). We have developed an *in vitro* assay system to measure AP site repair involving either pathway (15). AP sites are generated from unstable base modifications and also as common intermediate products following the first step of base-excision repair, in which damaged bases are removed by specific DNA-*N*-glycosylases. In this assay system, both of these pathways can repair natural AP sites, whereas tetrahydrofuran residues are repaired primarily by the proliferating cell nuclear antigen-dependent pathway. A ^{32}P-labeled plasmid carrying either lesion was incubated with extracts from treated cells and analyzed for its repair. The repaired products in this assay system should yield a 382-bp fragment, whereas the unrepaired DNA, which is still sensitive to AP endonuclease, would yield a shorter fragment. Fig. 1 shows that although extracts from untreated cells resulted in natural AP site repair in proportion to the amount of cell extract added, no increase in repair was observed in the extracts prepared following exposure of HT29 cells to 30 or 100 μM oltipraz. The repair of tetrahydrofuran residues was examined with similar results. Therefore, treatment with oltipraz did not induce base-excision repair activity in HT29 cells.

Treatment of cells with the chemotherapeutic drug cisplatin induces a variety of lesions in DNA. Platinum binding to DNA occurs initially as monoadducts, which may then be converted to either intrastrand or interstrand cross-links (23). The repair of platinum-DNA adducts is

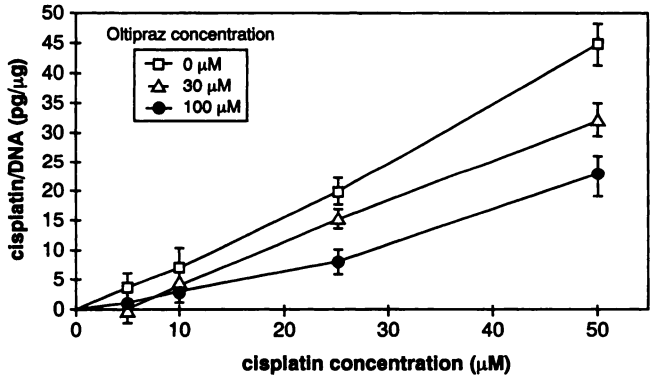

Fig. 2. Effect of oltipraz on the formation of platinum-DNA adducts in HT-29 cells. Cells were preincubated for 24 h with 0, 30, or 100 μM of oltipraz, followed by a 4-h exposure to a range of cisplatin concentrations (0–50 μM). DNA was isolated, and platinum content was determined by AAS as described in "Materials and Methods." Each *point* is the result of triplicate measurements made for each DNA sample; *bars*, SD.

[4] P. J. O'Dwyer *et al.*, submitted for publication.
[5] P. J. O'Dwyer and K-S. Yao, unpublished data.

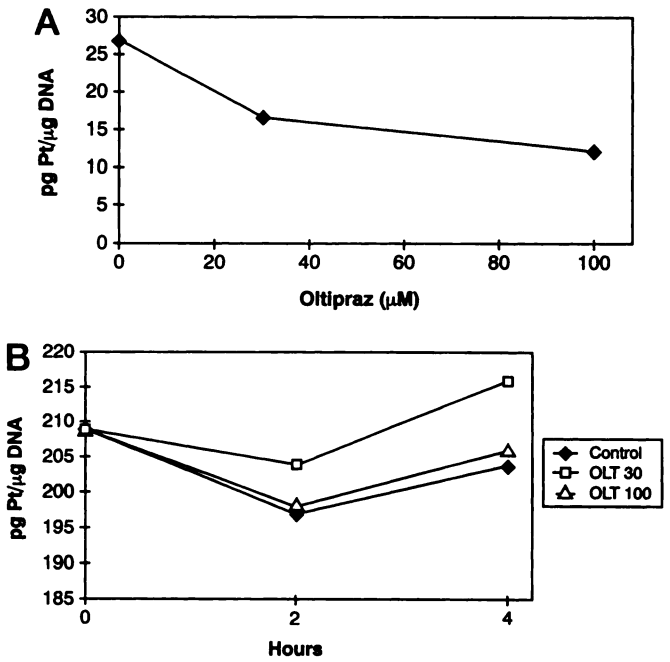

Fig. 3. Effect of oltipraz on the formation and removal of platinum-DNA adducts from purified HT-29 DNA. In *A*, cisplatin (10 ng) was incubated simultaneously with 50 μg of DNA and 0, 30, or 100 μM oltipraz for 4 h at 37°C. In *B*, purified DNA from cisplatin-treated HT-29 cells was incubated with 0, 30, or 100 μM of oltipraz for 0, 2, or 4 h at 37°C. Platinum content was determined by AAS as described in "Materials and Methods."

believed to occur primarily by NER (14). Several investigators have characterized previously the kinetics of formation and repair of various platinum-DNA adduct species in ovarian cancer cells (17, 24–28). In the present study, cisplatin-treated HT29 cells accumulated platinum-DNA adducts in a concentration-dependent manner (Fig. 2). Concomitant treatment with oltipraz decreases the rate and the peak accumulation of platinated DNA at concentrations ≥30 μM. This effect was also observed when oltipraz and cisplatin were concurrently incubated with purified DNA (Fig. 3*A*). These data suggest that oltipraz may act by directly quenching the ability of carcinogens or radicals to bind DNA, as well as by inducing enzyme-mediated inactivation. Furthermore, they do not rule out the possibility that oltipraz can displace platinum or carcinogens already bound to DNA. To examine this possibility, we incubated HT29 DNA with cisplatin followed by oltipraz. Fig. 3*B* shows that oltipraz failed to displace platinum from DNA directly. Also, oltipraz incubated with platinated plasmid DNA *in vitro* failed to displace the bound metal (data not shown). It is presently unclear whether oltipraz directly inactivates cisplatin or whether it interacts with DNA to mask or alter potential drug-binding sites. Preliminary studies in our laboratory have not yielded evidence for the formation of an oltipraz-cisplatin complex (data not shown).

In addition to measuring platinum binding to DNA, the effects of oltipraz on the repair/removal of total platinum-DNA adducts and interstrand cross-links were investigated. The effect of oltipraz on the repair of cisplatin-DNA adducts in HT29 cells was examined by treating cells with 100 μM oltipraz for 24 h, followed by exposure to a cisplatin concentration high enough to yield the same number of cisplatin-DNA adducts as were formed by exposure of the HT29 cells that were not treated with oltipraz to 50 μM cisplatin for 4 h. Under these conditions of equivalent DNA platination in oltipraz-treated and -untreated cells, a 3-fold enhancement of total platinum-DNA adduct removal was observed as measured by AAS (data not shown). An effect of oltipraz on DNA interstrand cross-link removal was also observed. Cross-links were measured in a constitutively expressed multicopy gene (rRNA) using the renaturating agarose gel electrophoresis assay, in which restriction-digested cross-linked DNA remains double-stranded under denaturing conditions and, therefore,

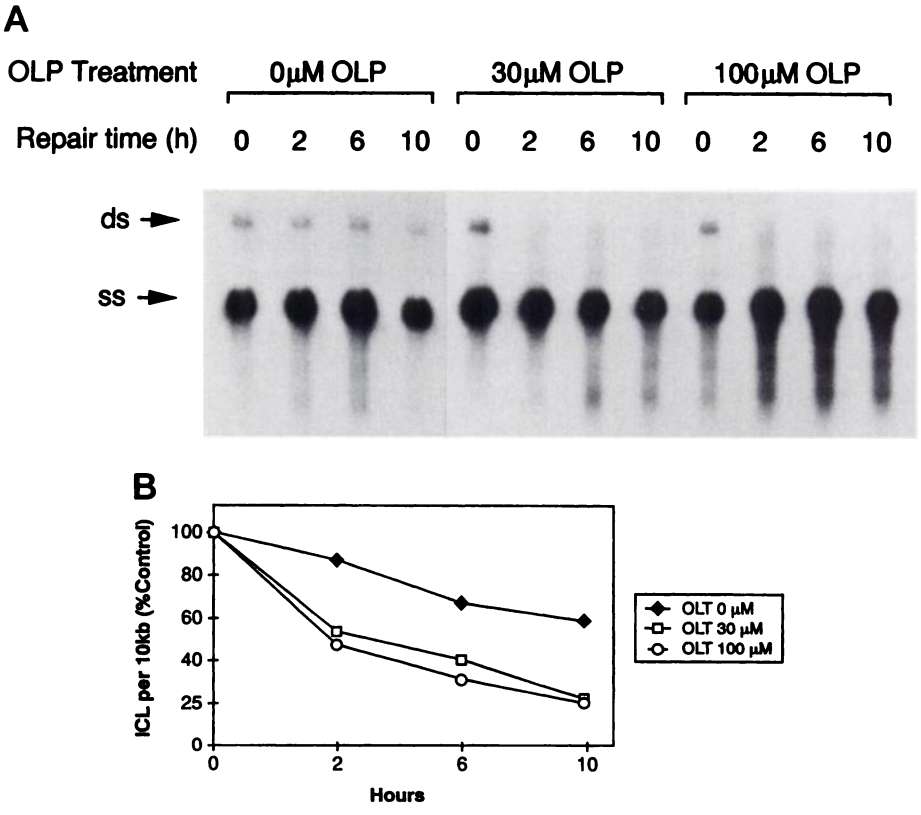

Fig. 4. Removal of cisplatin interstrand cross-links in HT-29 cells preincubated for 24 h with 0, 30, or 100 μM oltipraz. Cells were treated with cisplatin for 4 h at 37°C and either harvested immediately or incubated in drug-free medium for 2, 6, or 10 h. DNA was isolated and subjected to renaturating agarose gel electrophoresis (*A*) as described in "Materials and Methods." Cross-links were quantitated as described in "Materials and Methods" (*B*).

may be recognized and quantitated as a slower migrating band in Southern blots. Oltipraz concentrations ≥30 μM resulted in fewer cross-links and in their more rapid removal (Fig. 4). It is believed that although some of the effect of oltipraz could be a consequence of diminished adduct formation as was observed *in vitro,* most is a result of an effect on DNA repair, because we and others have found that increased intrastrand cross-link formation following cisplatin removal does not occur (28, 29).

These findings suggest that the chemoprotective drug oltipraz may have a stimulating effect on NER but not on base excision repair. Because the carcinogens from which oltipraz protects animals share the capacity to form bulky DNA adducts, induction of the NER pathways are, therefore, a plausible mechanism of oltipraz action. Oltipraz has been shown to protect aflatoxin B_1-treated animals from hepatoma formation and from lung and forestomach tumors following benzo(*a*)pyrene exposure (5, 6). It also protects rats from colon tumor formation following azoxymethane (7). Although some of these carcinogens have the potential to generate free radicals and damage DNA indirectly, they have in common an ability to form bulky adducts, particularly to adenine and guanine residues. Our results indicate that in addition to inhibition of their formation, the repair of such adducts is stimulated by oltipraz. Furthermore, oltipraz may not itself have a role in the repair of lesions such as photoadducts that may result from radiation exposure; but given these results, a broader investigation of compounds capable of such protection is justified.

NER results from the coordinate action of over 40 proteins (30). Aboussekhra *et al.* (30) have recently purified all of the proteins involved and have reconstituted NER *in vitro*. The mechanism by which oltipraz may influence the rate of repair is unclear at present. One possibility is that oltipraz treatment results in the up-regulation of NER genes, as has been demonstrated for detoxication genes (31). Oltipraz may have the potential to alter redox characteristics intracellularly; however, the lack of an effect of oltipraz on adduct formation in a cell-free system argues against the importance of such an action. On the other hand, the finding of fewer adducts and cross-links in the immediate aftermath of oltipraz treatment suggests an additional direct effect. The induction of detoxicating pathways is not immediate; *in vitro* and *in vivo* studies demonstrate peak activities that are delayed for approximately 24 h after treatment. The induction of elevated transcription factor content and binding activities is more rapid, however, and may relate to inhibition of DNA adduct formation by oltipraz (19).

These results suggest an effect of oltipraz on the repair of DNA adducts following exposure to various DNA-damaging agents and may account for the observation that initiating oltipraz treatment after carcinogen exposure may be as protective as pretreatment, although such an effect has not been found to be universal. Hence, oltipraz may reduce cancer risk not only by directly limiting the damage produced by potential carcinogens or by inducing genes in which their products enzymatically enhance detoxication, but as our data show, by increasing the repair of carcinogen-associated DNA damage. The data support NER as the principal repair system involved, but it is yet to be clarified how the change in activity is accomplished. The data also support the initiation of chemoprotective strategies in high-risk patients even after a history of mutagen exposure. The stimulation of DNA repair may further justify trials of compounds of this nature in individuals found to have been exposed to industrial pollutants such as dioxin. As appropriate clinical end points are identified, such studies should be considered.

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