Inhibition of Mitogen-activated Protein Kinase Activity of Human Lymphocytes after Oral Administration of Oltipraz[1]

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Abstract

Several preclinical studies indicated that Oltipraz appears to be one of the most potent cancer chemopreventive agents. Pharmacological studies in humans provided substantial amounts of information related to doses and schedules. Oltipraz has been reported to induce phase II drug-metabolizing enzymes. However, its chemopreventive activity suggests that it may also interact with cellular processes associated with cancer cell growth and proliferation. During a clinical trial designed to monitor eventual Oltipraz toxicity in high-risk population for development of lung cancer, we performed companion studies related to cell proliferation. Human lymphocytes were chosen as surrogate tissue to assess the *in vivo* effects of Oltipraz on cell signaling pathways involved in cell proliferation. The results of this study demonstrate that Oltipraz markedly inhibited the activation state of the extracellular signal-regulated kinases of the mitogen-activated protein kinase family of kinases in lymphocytes of subjects treated with two different doses and schedules of Oltipraz. Individual variations were observed that were not related to Oltipraz dosing or schedule of administration. The results from this study indicate that lymphocytes could be used as surrogate tissue for the development of biomarkers for studies of anticarcinogenic agents.

Introduction

Oltipraz is a synthetic dithiolthione [4-methyl-5-(2-pyrazinyl)-1-2-dithiol-3-thione] which was introduced as an antischistosomiasis agent. It is structurally similar to the dithiolthiones present in a variety of cruciferous vegetables. Oltipraz has high lipid solubility and nonlinear pharmacokinetics, and its differential absorption is dependent on the fat content of the diet (1–3). Large numbers of metabolites have been reported in animals, but their presence in humans taking the drug is not well defined (4, 5). During the last two decades, it has also been evaluated as a cancer chemopreventive agent (1, 2, 6, 7). On the basis of extensive testing, Oltipraz was regarded as a promising chemopreventive agent with high overall preventive indices (7). Characterization of the activity of Oltipraz in animal models of chemically induced carcinogenesis indicated that it protects several target organs from a variety of carcinogens (8). The precise mechanism by which Oltipraz inhibits tumor formation is unknown, but it is generally accepted that the induction of several phase II detoxification enzymes is at least one of the anticarcinogenic effects of this drug (1, 7, 9). Apart from its ability to induce phase II detoxifying enzymes, the effect of Oltipraz as a chemopreventive agent also suggests that this agent may interfere with cellular components associated with cell proliferation, although such effects have not been demonstrated previously. The ERK[3] group of MAPKs are known to play a central role in the transduction of mitogenic signals that culminate in cell growth and proliferation (10). The tumorigenic action of many oncogenes such as *src, ras,* and others has been shown to involve activation of ERK/MAPKs (11, 12). In this communication, we report the results of companion studies done during the execution of a clinical trial designed to monitor eventual Oltipraz toxicity (13). We observed that Oltipraz administration inhibited the activation state of ERK/MAPKs of lymphocytes, which is a part of the RAS signal transduction pathway.

Materials and Methods

Study Subjects. Thirty subjects, 18 females and 12 males, all heavy smokers (>20 pack-years) ranging in age from 32 to 62 years, participated in this study. All subjects had abnormal sputum cytology at baseline. Normal chest X-ray, complete peripheral blood counts, and biochemical profiles were required by the eligibility criteria for participation in the study. Premenopausal women were required to have a negative pregnancy test before accepted into the study and to follow appropriate contraceptive measures while participating in the protocol. Before the study, a physician evaluated all subjects eligible to participate. In addition, the eligible subjects were required to sign an appropriate informed consent form approved by both the National Cancer Institute and the University Committee on Research Involving Human Subjects at Michigan State University according to the Helsinki declaration.

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[3] The abbreviations used are: ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase.

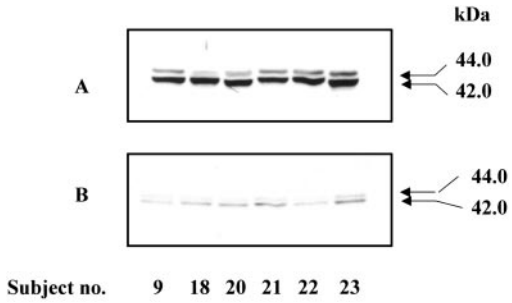

Fig. 1. Inhibition of ERK/MAPK activation in lymphocytes after Oltipraz treatment of the subjects. ERK/MAPK activation state of the lymphocytes was assessed by immunoblotting as described in "Materials and Methods." Shown here is a representative immunoblot of the phosphorylated ERK/MAPK in lymphocytes isolated before (*A*) and after (*B*) Oltipraz treatment of the subjects.

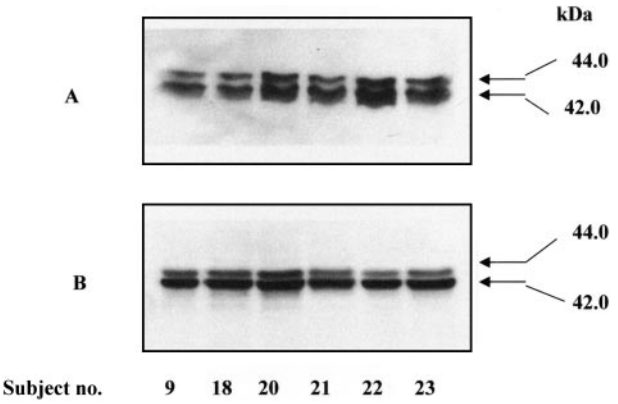

Fig. 2. Levels of total ERK/MAPK in lymphocytes after Oltipraz treatment of the subjects. The total ERK/MAPK level of the lymphocyte samples was determined by immunoblotting as described in "Materials and Methods." Lymphocyte samples before (*A*) and after (*B*) Oltipraz treatment of the same subjects used for determining ERK/MAPK activation state were also used for total ERK/MAPK levels.

Drug Supply. Oltipraz was provided by the Chemoprevention Branch, National Cancer Institute, and produced by the Rhône-Poulenc Company (Paris, France). The purity of the drug was >99% as determined by high-performance liquid chromatography and gas liquid chromatography-mass spectrometry. The drug was supplied in capsules containing 100 and 250 mg of Oltipraz in lactose.

Dose and Schedule Selection. On the basis of previous pharmacological trials, doses of 500 mg of Oltipraz once weekly for 30 days and 200 mg twice weekly for 30 days were given in this study and were considered safe and capable of modifying several biochemical markers (5, 8, 13). For the purpose of the present study, blood was collected before the administration of Oltipraz and 2 days after the last dosing.

Drug Administration. Subjects ingested Oltipraz p.o. at 7:30 a.m. with 180 ml of whole milk on an empty stomach. Milk was given to provide a uniform environment during the absorption of this lipid-soluble compound. The capsules were ingested in the presence of the drug distributor, assuring 100% compliance with protocol requirements.

Antibody Supply. The antibodies against phosphorylated (activated) ERK/MAP kinases and total ERK/MAP kinases (antibodies that recognize MAPKs independent of their phosphorylation state) were purchased from New England Biolabs, Inc. (Beverly, MA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. The enhanced chemiluminescence (ECL) Western blotting detection reagents were obtained from Amersham Biosciences Corp. (Piscataway, NJ).

Isolation of Lymphocytes. The isolation of lymphocytes from human blood, using the method of Büyum (14), is a standard procedure in our laboratory. The lymphocytes have a long life span, which makes them a suitable tissue for metabolic studies (15).

Biochemical Assay. The activation state of ERK/MAPK was determined using Western blot analysis essentially as described previously (16, 17). Briefly, 50 μg of protein from each lymphocyte sample were resolved on 10% SDS-PAGE gels and transferred to supported nitrocellulose membranes (17). The membranes were incubated with either phosphospecific ERK/MAPK (dilution, 1:500) or total ERK/MAPK (dilution, 1:10,000) antibodies, followed by incubation with peroxidase-conjugated secondary antibodies. The images of the ERK/MAPKs were obtained on X-ray film after treatment of the membranes with ECL reagents. The time of exposure of the membranes to the film was 1 min. Digital analysis of the intensity of the ERK/MAPK bands on the film was performed using UN-scan-it gel software (Silk Scientific, Orem, UT). The images on the X-ray films were scanned on a flatbed scanner, and the band intensity was digitized using Un-scan-it-gel software. The pixel values of the digitized bands were used to assess the levels of ERK/MAPK (activated or total). Two gels were used for immunoblotting with either activated ERK/MAPK or total ERK/MAPK to confirm the results. Representative blots of the gels are presented in Figs. 1 and 2.

Results

The clinical aspects of Oltipraz administration using the same schedules and doses were reported previously (13). To examine whether Oltipraz administration affects signaling pathways associated with cell proliferation of the surrogate tissue, we examined the activation state of ERK/MAPK in lymphocytes before treatment and 2 days after the last dose of Oltipraz administration in subjects of both treatment groups as described above. The blood concentrations of Oltipraz at the end of treatment were elevated in the majority of the subjects of both treatment groups. However, there were substantial interindividual variations of Oltipraz concentrations in both study groups, *i.e.,* 500 mg once weekly or 200 mg twice weekly (Tables 1 and 2).

The activation of ERK/MAPK was determined by the extent of phosphorylation of the ERK/MAPK in immunoblot analysis. As shown in Tables 1 and 2, the phosphorylation state of ERK/MAPK varied among the samples before the start of the Oltipraz treatment. In the group treated with 500 mg of Oltipraz weekly, lymphocytes from 9 subjects showed high levels of activated (phosphorylated) ERK/MAPK, whereas the remaining 6 had low levels of activated ERK/MAPK before the start of treatment (Table 1). The phosphorylation state of

Table 1 Comparison of serum Oltipraz concentrations[a] to level of phosphorylated ERK/MAPK (LPERK/MAPK) in lymphocytes[b]

Registration no.	LPERK/MAPK[c] Day 0	LPERK/MAPK Day 30	Serum Oltipraz, ng/ml Day 30
1	12571	8500	0.5
2	9875	8500	0
3	14800	12000	0.6
4	10500	10000	0
5	12850	15800	0.4
6	13800	12000	0
13	3400	3300	24.0
14	4375	3800	30.0
15	5600	2980	6.0
17	4200	7000	1.1
19	4350	7980	0
24	8000	4250	2.0
25	7100	4250	7.0
26	8250	2100	1.5
27	5065	3260	1.9

[a] Subjects treated with 500 mg of Oltipraz once a week for 4 weeks.
[b] Lymphocytes were collected before treatment and 2 days after the last dose.
[c] Pixels.

Table 2 Comparison of serum Oltipraz concentrations[a] to level of phosphorylated ERK/MAPK (LPERK/MAPK) in lymphocytes[b]

Registration no.	LPERK/MAPK[c] Day 0	LPERK/MAPK Day 30	Serum Oltipraz, ng/ml Day 30
7	17050	4200	9.0
8	18200	2950	0
9	22100	2570	0
10	15070	2050	0.6
11	12850	3200	18.0
12	12000	3365	10.0
18	21000	3500	5.0
20	24500	4000	0
21	25000	4100	0.8
22	21500	4850	0
23	20000	4600	0.5
28	9246	4800	2.5
29	7382	4300	0
30	11250	1100	8.0
31	12531	1537	3.0

[a] Subjects were treated with 200 mg of Oltipraz twice a week for 4 weeks.
[b] Lymphocytes collected before treatment and 2 days after the last dose.
[c] Pixels.

ERK/MAPK in lymphocytes of these 9 subjects was significantly decreased at the end of the treatment. The total ERK/MAPK levels of lymphocytes from patients in this group appeared to remain at the same level as the pretreatment level (data not shown), which indicated that the changes in the phosphorylation state of the ERK/MAPKs was not attributable to a reduction in the level of total ERK/MAPK protein. The 15 subjects who received Oltipraz twice a week at 200 mg/kg also showed variations in the level of activated ERK/MAPK (Table 2) before treatment. In 11 subjects of this group, the level of phosphorylated ERK/MAPK at the start of the treatment was higher than the remaining 4 subjects. As shown in Table 2 and represented in Fig. 1*B,* the phosphorylation state of ERK/MAPK after treatment was markedly reduced in lymphocytes obtained from the 11 subjects. In the remaining 4 subjects, the phosphorylation state of ERK/MAPKs was low before the start as well as at the end of Oltipraz treatment (Table 2). To determine whether the observed difference in the activated ERK/MAPK levels of the 11 subjects of the 200 mg of Oltipraz twice a week group was attributable to changes in the levels of ERK/MAPK proteins, we performed immunoblot analysis for total ERK/MAPK levels of lymphocytes from the same subjects. As represented in Fig. 2, the levels of total ERK proteins at the end of the treatment did not significantly differ in these subjects from the levels at the start of treatment. This suggested that Oltipraz treatment in these subjects affected the activation state of ERK/MAPK but did not alter the levels of these proteins. Comparing the level of phosphorylated ERK/MAPK proteins with serum concentrations of Oltipraz at day 30, we observed no correlation between those two parameters, suggesting no dose dependency.

Discussion

Oltipraz shows promising anticancer activity, but the mechanisms of its action are largely unknown. In this study, we have shown that Oltipraz treatment interferes with cell signal transduction pathways that play a central role in cell proliferation. The ERK is a member of the group of kinases that are mitogen-activated protein kinases (10). These kinases are important in the transduction of mitogenic signal from cell surface to the cell nucleus (12). Aberrant activation of ERK/MAPK has been associated with neoplastic transformation of cells (18). The phosphorylation state (activation) of ERK/MAPK is known to be regulated by many upstream components, such as tyrosine kinases, oncogenic GTP-binding RAS proteins, protein kinase C, and others. It is conceivable that aberrant regulation of the upstream regulators of the MAPK cascade can perturb the activation state of the MAPKs (11, 12, 19, 20). Many pharmacological agents that are being developed as potential anticancer agents are targeted to inhibit MAPKs (11, 21). Although Oltipraz is not expected to be a drug that inhibits the MAPK proteins, it is surprising that it inhibited the activation state of the ERK/MAPKs in lymphocytes from a majority of the subjects who took the drug. The differences in the response of lymphocytes could be explained with the difference in the circulating lymphocytes. Those differences could be related to the type of cells, the length of life span, and the age of the individual cells (15). However, the inhibition is so pronounced that the intercellular differences could not have influenced the biological outcome. Another explanation may be the response to dosing. The twice-a-week dose may result in a higher intracellular concentration of the drug, which could result in a greater inhibition. It has been shown recently that Oltipraz is an agonist of the arylhydrocarbon receptor, which also functions as a transcription factor that regulates the expression of cytochrome P-450 1A1 drug-metabolizing enzyme (22). It is unclear how the effects of Oltipraz on the induction of the detoxifying enzymes are related to down-regulation of ERK/MAPKs of lymphocytes. One may speculate that this drug may indirectly exert antioxidant effects and inhibit ERK/MAPK activity or indirectly alter the function of the upstream

regulators of the ERK/MAPK, leading to the inhibition of the latter. Clearly more studies are necessary to identify the effects of Oltipraz on signal transduction pathways and the mechanisms of such an effect.

Surrogate tissue such as lymphocytes are not the target tissue for the chemopreventive activity of Oltipraz. However, it may represent the biochemical and molecular alterations attributable to the drug treatment. Moreover, such tissue could be obtained without invasive procedures and assayed for biomarker changes induced by the investigative drugs.

In summary, the results of the present study indicate a new pharmacological activity of Oltipraz, which may be related to its cancer chemopreventive/anticarcinogenic effect. Although this observation was based on a small sample, it clearly suggests the need for further investigation.

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References

1. Wattenberg, L. W. Chemoprevention of Cancer. Cancer Res., *45:* 1–8, 1985.

2. Dimitrov, N. V., Bennett, J. L., McMillan, J., Perloff, M., Leece, C. M., and Malone W. Clinical pharmacology studies of Oltipraz—a potential chemopreventive agent. Investig. Drugs, *10:* 289–298, 1992.

3. Benson, A. B., III. Oltipraz: a laboratory and clinical review. J. Cell Biochem., *17:* 278–291, 1993.

4. Kensler, T. W., and Helzlsouer, K. J. Oltipraz. Clinical opportunities for cancer chemoprevention. J. Cell Biochem., *22:* 101–107, 1995.

5. Gupta, E., Olopade, O. I., Ratain, M. J., Mick, R., Baker, T. M., Berezin, F. K., Benson, A. B., III, and Dolan, M. E. Pharmacokinetics and pharmacodynamics of Oltipraz as a chemopreventive agent. Clin. Cancer Res., *1:* 1133–1138, 1995.

6. Bieder, A., Decouvelaere, B., Gaillard, C., Depaire, H., Heusse, D., Ledoux, D., Lemar, M., LeRoy, J. P., Raynaud, L., Snozzi, C., and Gregoire, J. Comparison of the metabolism of Oltipraz in the mouse, rat, monkey and in man. Arzneim.-Forsch., *33:* 1289–1297, 1983.

7. O'Dwyer, P. J., Szarka, C. E., Yao, K. S., Halberr, T. C., Pfeiffer, G. R., Green, F., Gallo, J. M., Brennan, J., Fracht, H., Goosenberg, E. B., Hamilton, T. C., Litwin, S., Balshem, A. M., Engstorm, P. F., and Clapper, M. L. Modulation of gene expression in subjects at risk for colorectal cancer by the chemopreventive agent dithiolethione Oltipraz. J. Clin. Investig., *98:* 1210–1217, 1992.

8. Kelloff, G. J., Bodne, C. W., Crowell, J. A., Steel, V., Lubet, R., and Sigman, C. C. Chemopreventive drug development: perspectives and progress. Cancer Epidemiol. Biomark. Prev., *3:* 85–98, 1994.

9. Manson, M. M., Ball, H. W., Barrett, M. C., Clark, H. L., Judah, D. J., Williamson, G., and Neal, G. E. Mechanism of action of dietary chemopreventive agents in rat liver: induction of Phase I and II drug metabolizing enzymes and aflatoxin B1 metabolism. Carcinogenesis (Lond.), *18:* 1729–1738, 1997.

10. Gudermann, T. Multiple pathways of ERK activation by G protein-coupled receptors. Novartis Found. Symp., *239:* 68–79, 2001.

11. Ramirez-De-Molina, A., Rodriguez-Gonzalez, A., and Lacal, J. C. Targeting new anticancer drugs within signaling pathways regulated by Ras GTPase superfamily. Int. J. Oncol., *19:* 5–17, 2001.

12. Amundadottir, L. T., and Leder, P. Signal transduction pathways activated and required for mammary carcinogenesis in response to specific oncogenes. Oncogene, *16:* 737–746, 1998.

13. Dimitrov, N. V., Leece, C. M., Tompkins, E. R., Seymour, E., Bennink, M., Gardiner, J., Crowell, J., Hawk, E., Nashawaty, M., and Bennet, J. Oltipraz concentrations in plasma, buccal mucosa cells, and lipids: pharmacological studies. Cancer Epidemiol. Biomark. Prev., *10:* 201–207, 2001.

14. Böyum, A. Separation of leukocytes from blood and bone marrow. Scand. J. Clin. Lab. Investig., *21* (Suppl. 97)*:* 45–47, 1968.

15. Carson, D. A. Composition and biochemistry of lymphocytes and plasma cells. *In:* E. Beutler, M. A. Lichtman, B. S. Coller, and T. J. Kipps (eds)., Williams Hematology, 5th Ed, pp. 916–921. New York: The McGraw Hill Companies, 1990.

16. Fischer, L. J., Wagner, M. A., and Madhukar, B. V. Potential involvement of calcium, CaM kinase II, and MAP kinases in PCB-stimulated insulin release from RINm5F cells. Toxicol. Appl. Pharmacol., *159:* 194–203, 1999.

17. Towbin, H., Staehelin, T., and Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA, *76:* 4350–4354, 1979.

18. Robinson, M. J., and Cobb, M. H. Mitogen-activated protein kinase pathways. Curr. Opin. Cell Biol., *9:* 180–186, 1997.

19. Cobb, M. H., and Goldsmith, E. J. How MAP kinases are regulated. J. Biol. Chem., *270:* 14843–14846, 1995.

20. Kolch, W. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem. J., *351:* 289–305, 2000.

21. Sebolt-Leopald, J. S. Development of anticancer drugs targeting the MAP kinase pathway. Oncogene, *19:* 6594–6599, 2000.

22. Carr, B. A., and Franklin, M. R. Induction of drug metabolizing enzymes by 1,7-phenanthroline and Oltipraz in mice is unrelated to Ah-responsiveness. J. Biochem. Mol. Toxicol., *13:* 77–82, 1999.