Effects of Oltipraz and Related Chemoprevention Compounds on Gene Expression in Rat Liver

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Abstract One promising approach to cancer chemoprevention involves the induction of enzymes of phase II xenobiotic metabolism. Since this approach requires use of drugs specifically intended to alter tissue gene expression patterns over long periods, it will be important to determine experimentally which proteins are increased or decreased by treatment, and how such alterations may (or may not) be related to the postulated chemopreventive mechanism. We have employed two-dimensional electrophoresis as a tool to detect and quantitate gene expression effects of candidate chemoprevention compounds in the livers of treated rats. Oltipraz, an inducer of several phase II enzymes, was shown to affect a series of at least 26 proteins, most of which were slightly decreased by treatment. Several proteins were increased, the prime example being rat liver spot 693, which was induced more than 7-fold by oltipraz. This protein was excised from multiple 2-D gels and subjected to *in situ* tryptic digestion followed by microchemical sequence analysis. The resulting multiple peptide sequences match perfectly with the cDNA derived sequence of rat aflatoxin B_1 aldehyde reductase (AFAR). Using quantitative measurements of AFAR from 2-D gels, we compared a series of dose regimens. Oltipraz administration by gavage or in diet appeared equally effective, while recovery studies indicated a half-time of 5.5 days for disappearance of the AFAR protein. Oltipraz analogs anethole trithione (ANTT), 1,2-ditiole-3-thione (1,2-DT-3-T) and 1,3-dithiole-2-thione (1,3-DT-3-T) were examined with respect to ability to increase liver AFAR levels: ANTT appeared approximately equipotent with oltipraz, 1,2-DT-3-T appeared more than 10 times as potent, and 1,3-DT-2-T did not significantly induce AFAR, while nevertheless causing significant changes in a distinct set of proteins. This latter set was shown, by multivariate statistical comparison with an extended set of chemoprevention compounds, to closely resemble the effects of piroxicam at high dose.

Key words: aflatoxin B_1 aldehyde reductase, gene expression, oltipraz, piroxicam, 2-D electrophoresis

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Running Title: Gene Expression Effects of Oltipraz

A major strategy for cancer chemoprevention involves the introduction of natural or synthetic agents into the diet to lower cancer incidence. The possibility that diet could indirectly modify cancer induction arose in the 1930's when it was observed that vitamin B deficiency enhanced liver cancer induction by ρ-dimethyl [1], and recently a series of promising leads have been developed aimed at human cancer prevention. Dietary calcium glucarate shows chemopreventive activity against azoxymethane-induced rat colon tumors [2]. Cruciferous vegetables containing a variety of substituted dithiolethiones have been shown epidemiologically to be potent in inhibiting the development of several types of cancer, including human colon cancer [3-5]. Many such agents exert their anticancer action by inducing or inhibiting enzymes (proteins) involved in various metabolic functions of the cell. A class of substituted dithiolethiones, one member of which is 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione (oltipraz), is presumed to exert its anticancer action by inducing enzymes involved in electrophile detoxification, such as glutathione S-transferase, epoxide hydrase, and NAD(P)H-quinone oxidoreductase [6,7]. In the case of aflatoxin B_1-induced hepatocarcinogenesis, the anticarcinogenic action of dithiolethiones was due to alterations in the activity of both phase I and phase II enzymes in the liver [8,9]. Another dithiolethione, anethole trithione, has properties similar to those of oltipraz, and has been shown to raise GSH (reduced glutathione) levels in liver, lung, kidney, and upper jejunal mucosa following a single intragastric dose of 2 to 4 mmole/kg. Quinone reductase was similarly increased in the liver, lung, and upper jejunal mucosa [10]. Recently, administration of oltipraz and anethole trithione was found to be protective against the toxic effects of

acetaminophen [11] and carbon tetrachloride [12]. Mortality and liver damage in mice given these hepatotoxic compounds was significantly reduced in pretreated animals [10,12]. Many, if not most of these effects are attributable to alterations in the levels of specific tissue proteins as a result of treatment with a chemopreventive drug.

Two-Dimensional Electrophoresis

Two-dimensional electrophoretic protein mapping [13,14] is well suited to the detection and quantitation of such changes, because of its ability to separate more than 1,000 protein species in samples of unfractionated mammalian liver. The method makes use of sequential perpendicular separations by isoelectric focusing and SDS gel electrophoresis to give a 2-D map of proteins, which can then be stained with a protein-binding dye such as Coomassie Brilliant Blue, digitized and reduced to quantitative data through use of a computer [15]. In recent years, the effects of a broad range of xenobiotics have been studied in rodent liver by this approach, including chlorinated hydrocarbons [16], methapyrilene (a novel non-genotoxic carcinogen) [17], ibuprofen and phenobarbital [18], cholesterol synthesis inhibitors [14], and peroxisome proliferators [19]. In parallel, the growth of sequence databases and improvements in protein sequencing technique have made possible rapid strides in the identification of protein spots observed on 2-D gels. By combining 2-D analytical technology with a database of drug effects fingerprints and protein spot identifications, it is possible to examine the effects of new compounds with a reasonable expectation of classifying the mechanism and identifying some of its principal biochemical components.

In the context of chemoprevention, this approach could establish patterns of change specific to an agent or class of agents by identifying major responsive proteins

indicative of the associated mechanism of action *in vivo*. Further, the up-regulation or down-regulation of a set of specific proteins could provide new markers for determining the efficacy of a chemopreventive agent in additional *in vivo* tests. Liver, being the major metabolically active organ, is an ideal tissue to study the effects of chemopreventive agents.

Effects of Oltipraz on the 2-D Protein Pattern of Rat liver

Oltipraz causes quantitative changes in a series of liver proteins following exposure of 6-8 week old male F-344 rats to doses in the range 10-100 mg/kg (Fig 1). The twenty-six spots shown were selected as demonstrating a $P<0.001$ quantitative t-test difference from controls following either 4 days of 100 mg/kg gavage dosing or 4 days of 1000ppm dosing in feed. Of these proteins, two had been identified previously: spot 55 is the senescence marker protein SMP-30 (which decreased) and spot 87 is cytochrome b_5 (which is induced slightly under the same conditions). Overall, 17 proteins showed decreases with treatment (in the range 10-30%), while the remaining 9 proteins were increased. Three proteins (spots 693, 866 and 1177) were induced more than 2-fold, and one of these (693) is induced more than 7-fold. This pattern of gene expression change was consistent across the various oltipraz treatment regimens examined, and did not resemble the effects of any compound previously studied by 2-D protein mapping.

Identification of the Protein 693 Induced by Oltipraz

Rat liver protein 693 is more strongly induced by oltipraz treatment than any other protein detectable on standard wide range O'Farrell-type 2-D gels (covering an

isoelectric point range of 3.7-6.8). In order to identify this protein, the spot was excised from 80 stained 2-D gels and subjected to *in situ* digestion with trypsin. The resulting peptides were resolved by reverse-phase HPLC and 6 were sequenced using an Applied Biosystems protein sequencer at initial yields of 30-110 pmol. The sequence data (Fig. 2) shows complete identity with the sequence of rat aflatoxin B1 aldehyde reductase deduced from the sequence of a cloned cDNA (AFAR; ref 20). Isoelectric point and molecular weight computed from the whole sequence (pI=6.67, MW=36,681) correspond to the values observed for spot 693 in the 2-D patterns (pI=6.78, MW=32,600), confirming that the spot is AFAR.

Dose Response to Oltipraz in Different Treatment Protocols Using AFAR as a Biomarker

Using AFAR (spot 693) as a biomarker of gene regulation response to oltipraz, we evaluated a series of dosing regimens with respect to their ability to induce the enzyme (Fig. 3). In short treatment protocols (4 days), no significant difference was observed between gavage and dietary dosing. This result suggests that AFAR induction provides a measurement of the time-average oltipraz effect, and is unlikely to involve rapid changes associated with a short half-life protein.

Untreated recovery periods of three durations were evaluated (10 days after a 4 day gavage treatment, and 2 and 56 days after a 21 day dietary exposure) in order to determine the rate at which gene expression returned to control levels. AFAR abundance decreased with a half-time of approximately 5.5 days after the last oltipraz dose, indicating either that the protein has an equivalent half-life in the liver, or that oltipraz remains available and active in inducing its expression for an extended period.

Comparison of 4 and 21 day dietary exposures showed that the longer protocol induced final levels of AFAR that were only 60-70% as great as those achieved in short exposure. This result suggested the possibility that the induction of AFAR, and potentially other phase II enzymes, declines after longer term continuous dosing, perhaps due to an adaptive response by the liver. If this were the case, then an intermittent regime, with doses spaced so as to allow a decline in AFAR levels between doses, could be more effective in achieving a long term induction.

As a test of this hypothesis, four dosing frequencies were compared over 8 and 36 day durations with 30mg/kg administered in each dose (Fig. 4). The two durations produced very similar results for all frequencies (except for one unexplained aberrant point: triweekly 36day), indicating a general absence of a significant decline in response between one and five weeks. For both durations, response appears to increase monotonically with cumulative dose; however the intermittently delivered lower cumulative dose regimens (e.g., 180 mg/kg/36days, delivered as 30mg/kg once weekly) may yield slightly higher induction of AFAR (~2.7-fold) than the same cumulative dose delivered in equal daily aliquots (~1.5-fold computed from data of Fig. 3).

Comparison of Oltipraz with other Dithiolethiones

In order to investigate the structural specificity of oltipraz's induction of protein changes in rat liver, a series of three additional dithiolethiones was examined: anethole trithione (ANTT); 1,2-ditiole-3-thione (1,2-DT-3-T) and 1,3-dithiole-2-thione (1,3-DT-3-T). Dimethyl fumarate (DMF) was included as a negative control. Groups of 5 animals were treated by gavage for 4 days at equimolar doses equivalent to oltipraz dose levels of 100, 30, 10 and 0 mg/kg/day in corn oil vehicle. The results have been

analyzed in two ways (Figs. 5 and 6). In Figure 5, quantitative increases in AFAR are plotted for each of the five compounds as a function of dose. Neither DMF nor 1,3-DT-2-T elicit detectable increases in AFAR, while oltipraz and ANTT appear to be very nearly equally potent on a molar basis. However, the largest inductions, measuring up to 35-fold, are caused by 1,2-DT-3-T, the bare dithiolethione nucleus from which oltipraz is derived by addition of two substituent groups. At the lowest dose tested (44 μmol = 5.9 mg/kg/day), 1,2-DT-3-T treatment caused AFAR levels to rise 14-fold, exceeding the largest inductions seen with oltipraz or the other dithiolethiones at doses up to 100 mg/kg/day. As in earlier experiments, the dose response curve did not appear to be linear with dose, suggesting that the regulatory system controlling AFAR levels may be saturable.

Comparison of Oltipraz and its Analogs with other Chemoprevention Agents

Despite the low level of AFAR induction caused by 1,3-DT-2-T, it did cause a series of protein changes different in character from those caused by oltipraz. These changes were similar to those produced by high dose treatment with piroxicam, another potentially valuable chemoprevention compound. This relationship is apparent in the results of a multivariate statistical analysis using the principal components approach (Fig. 6). Principal axes were computed using a data set consisting of measurements of 81 proteins drawn from studies of oltipraz and its analogs, together with DMF and seven other candidate chemoprevention agents: ibuprofen, difluoromethyl ornithine (DFMO), N-(4-hydroxyphenyl)-retinamide (4-HPR), carbenoxolone, piroxicam, calcium glucarate, and 16α-fluoro-5α-androstane-17-one (a 16-fluoro DHEA analog). The position of each

experimental animal's protein expression pattern can be plotted on these axes (one letter symbol per animal) in order to visualize the relationships among the complex gene expression pattern changes associated with *in vivo* drug effects. The first component (labeled Factor 1) represents a pattern of abundance changes in these proteins associated with exposure to 1,3-DT-2-T (group "R") and piroxicam (group "P"): in each case the five experimental animals form a separate group displaced vertically from the cloud of control animal patterns (lower case letters near the center of the plot). Factor 2, the second largest overall pattern of quantitative change, is associated with oltipraz and its analogs: here 1,2-DT-3-T (group "W"), oltipraz (group "O2") and ANTT (group "A") are all clearly separated from the control group. However this representation, based on many protein effects instead of a single AFAR measurement, indicates that ANTT and oltipraz do not produce effects of equal magnitude, and that 1,2-DT-3-T is, overall, only about twice as potent as oltipraz in displacing the gene expression pattern away from the control values. This result suggests that the effective dithioledithiones may have differential effects on abundances of different liver proteins, with AFAR among the most responsive. Inspection of additional, smaller components indicates that factor 4 separates 4-HPR from the controls, while factor 5 further separates the effects of 1,3-DT-2-T and piroxicam (data not shown).

The distinct nature of the liver gene expression changes caused by oltipraz, piroxicam and 4-HPR (Table 1) are even more clearly revealed in a comparison of the specific proteins involved (here defined as those proteins showing a $P<0.001$ t-test difference between treated and appropriate control groups). Oltipraz and piroxicam show no overlaps, while 4-HPR has one protein in common with each of the former two sets.

Of the other compounds examined, only carbenoxolone shares an affected protein with any other compound. The single protein affected by ibuprofen (spot 367 which is increased) is known from previous studies to be strongly induced by peroxisome proliferators (manuscript in preparation) and to be anti-synergistically induced by two cholesterol-lowering treatments (lovastatin and cholestyramine, ref 14). This protein, whose sequence appears not to be in current sequence databases, is probably involved in some aspect of lipid metabolism.

Conclusions

Oltipraz and its analogs cause changes in the gene expression (protein abundance) pattern of rat liver in line with their relative potencies as chemopreventive agents. The most strongly induced protein among the subset resolved in this study was identified as aflatoxin B_1 aldehyde reductase, an enzyme likely to play an important role in disposal of at least one potent liver carcinogen. These results, taken together, provide additional support for the notion that global monitoring techniques (such as 2-D electrophoresis) provide important hypothesis-independent tools for exploring drug mechanisms. While almost all drugs appear to cause gene expression effects, the nature of the changes is likely to receive special scrutiny for those classes where the effect is intentional, forming part of the proposed mechanism of action. Chemoprevention compounds, particularly the phase II inducers, are thus ideal candidates for a comprehensive analysis of *in vivo* gene expression effects.

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21. Table Legend

Table 1: Comparison of sets of proteins showing quantitative changes (at $P<0.001$) following treatment with a series of candidate chemoprevention compounds.

Figure Legends

Fig.1 A schematic representation of the standard F344 rat liver 2-D protein pattern (ref 14,open ellipses), with a set of 26 protein spots affect by oltipraz shown as filled ellipses (each numbered with corresponding master spot number). A few identified proteins are labeled for reference: CPS is carbamyl phosphate synthase, FABP is the liver fatty acid binding protein, and the others are as indicated. Identified proteins whose abundances were affected by oltipraz treatment are shown with bordered labels. Acidic isoelectric points are to the left, basic to the right, and high molecular weights at the top, low MW at the bottom.

Fig. 2 Sequences of four internal tryptic peptides comprising 43 amino acids obtained from rat liver spot 693 (bold letters) aligned with the peptide sequence of rat aflatoxin B_1 aldehyde reductase as derived from a sequenced cDNA [20].

Fig. 3 Comparison of six oltipraz dosing regimens in terms of the abundance of aflatoxin B_1 aldehyde reductase in the liver (measured as integrated absorbance of spot 693 on Coomassie Blue stained 2-D gels of liver homogenates) at the end of treatment. Each point represents the average value for a group of 5 animals. Doses delivered in diet were computed based on weight of food actually consumed. A high dose 21-day diet treatment group was lost.

Fig. 4 Effect of the frequency of oltipraz administration on the level of liver aflatoxin B_1 aldehyde reductase (measured on 2-D gels) at the end of treatment. Each animal (5 per treatment group) received 30 mg/kg oltipraz per dose by gavage, and was sacrificed 24 hours following the last dose.

Fig. 5 Dose response curves for oltipraz, three other dithiolethiones and DMF in terms of relative AFAR induction

Fig. 6 Principal components analysis of the effects of a series of drugs on the abundance of 81 liver proteins selected as showing a $P<0.001$ significant difference from controls for at least one of the compounds in some regimen. Each symbol plotted represents an individual treated animal. Ellipses define major groups defined by the analysis. Factor 1 accounts for 16.7% of total data variance, while factor 2 accounts for 10.5%.

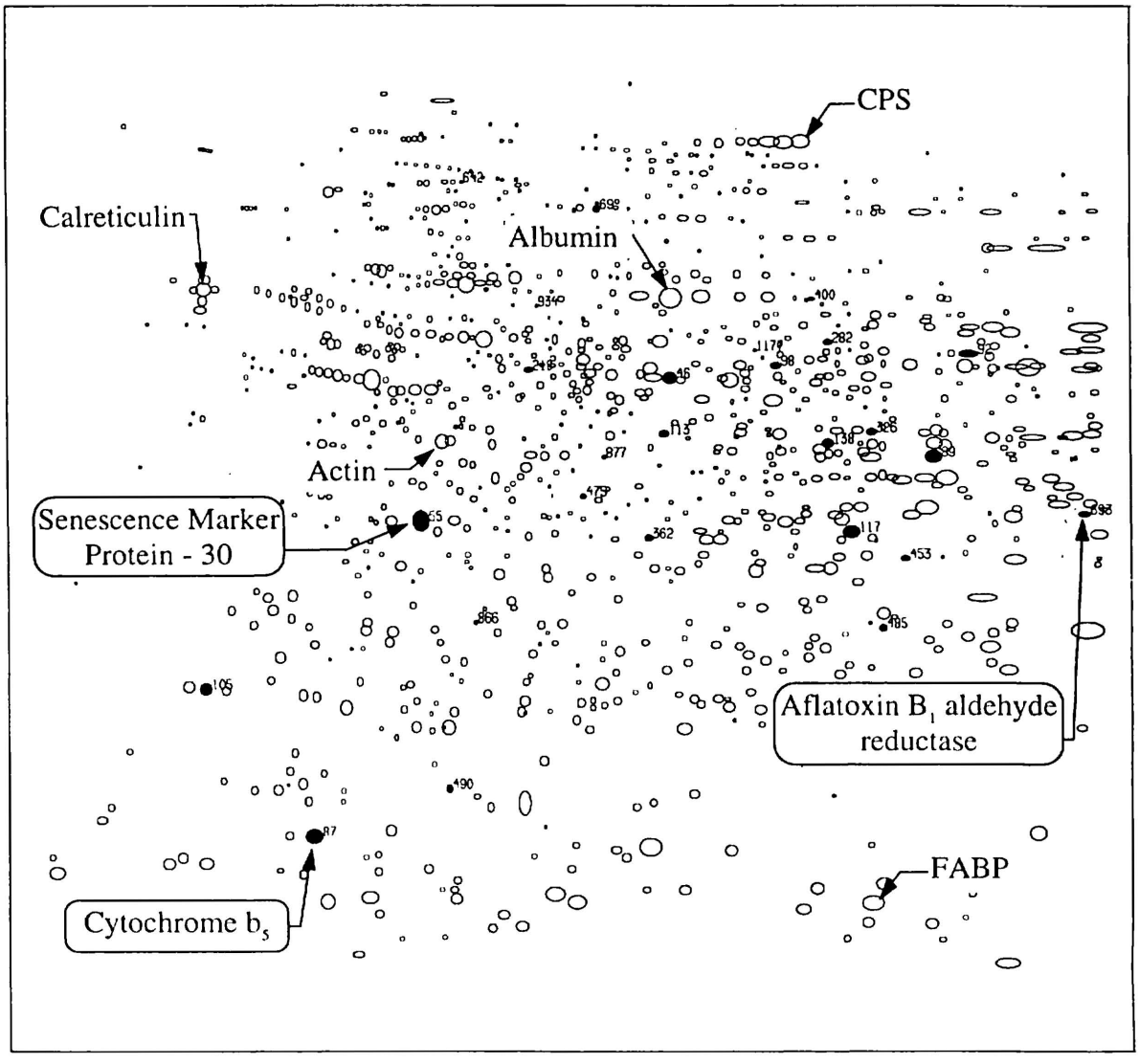

CPS
Calreticulin
Albumin
Actin
Senescence Marker Protein - 30
Aflatoxin B1 aldehyde reductase
FABP
Cytochrome b5
Experiment OLTI_SAR
17:39 16-OCT-94

Fig. 1

Identification of Rat Liver Spot 693 by Sequence of Internal Tryptic Peptides

(gp | X74673 | RNAFAR_1 aflatoxin B1 aldehyde reductase [Rattus norvegicus])

MSQARPATVLGAMEMGRRMDVTSSSASVRA
FLQRGHTEIDTAFVYANGQSETILGDLGLG
LGRSGCKVKIATKAAPMFGKTLKPADVR**FQ**
LETSLKRLQCPRVDLFYLHFPDHGTPIEET
LQACHHVHQEGKFVELGLSNYVSWEVAEIC
TLCKKNGIMPTVYQGMYNAITRQ**VETELFP**
CLRHFGLRFYAFNPLAGGLLTGRYKYQDKD
GKNPESRFFGNPFSQLYMDRYWKEEHFNGI
ALVEKALK**TTYGPTAPSMISAAVR**W**MYHHS**
QLKGTQGDAVILGMSSLEQLEQNLALVEEG
PLEPAVVDAFDQAWNLVAHECPNYFR

Fig 2.

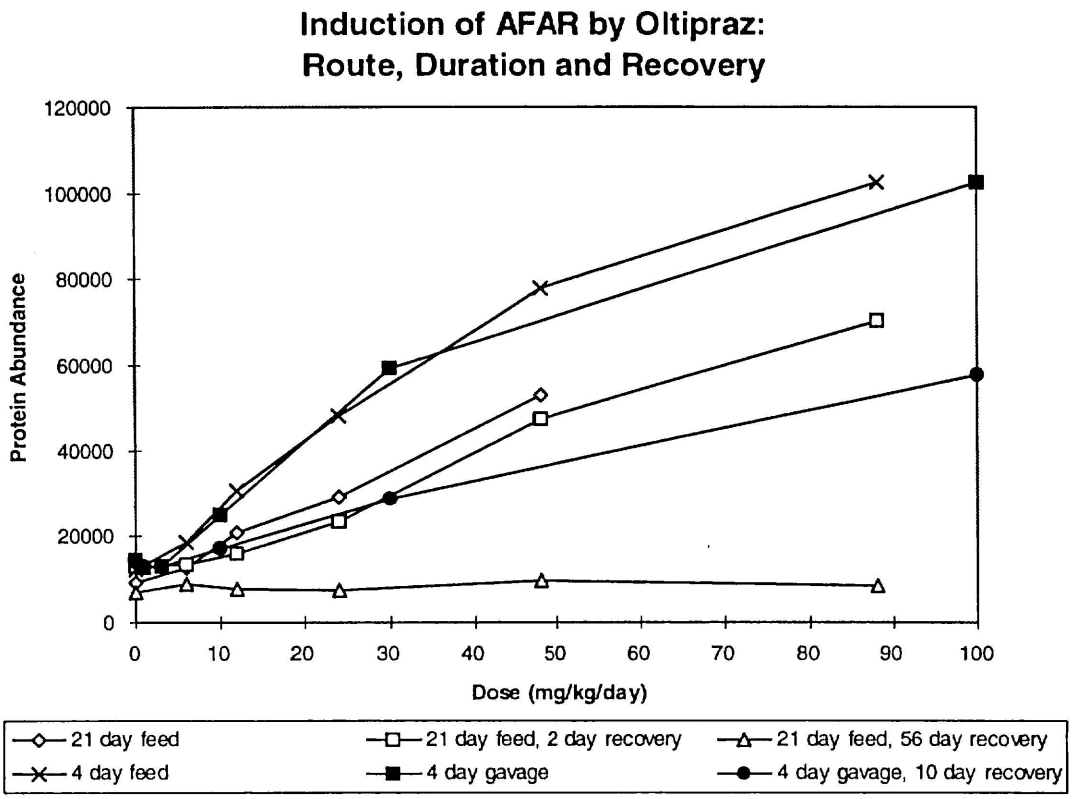

Induction of AFAR by Oltipraz:
Route, Duration and Recovery
Protein Abundance
120000
100000
80000
60000
40000
20000
0
0
10
20
30
40
50
60
70
80
90
100
Dose (mg/kg/day)
21 day feed
21 day feed, 2 day recovery
21 day feed, 56 day recovery
4 day feed
4 day gavage
4 day gavage, 10 day recovery

Fig. 3

Effect of Dosage Regimens on Induction of AFAR

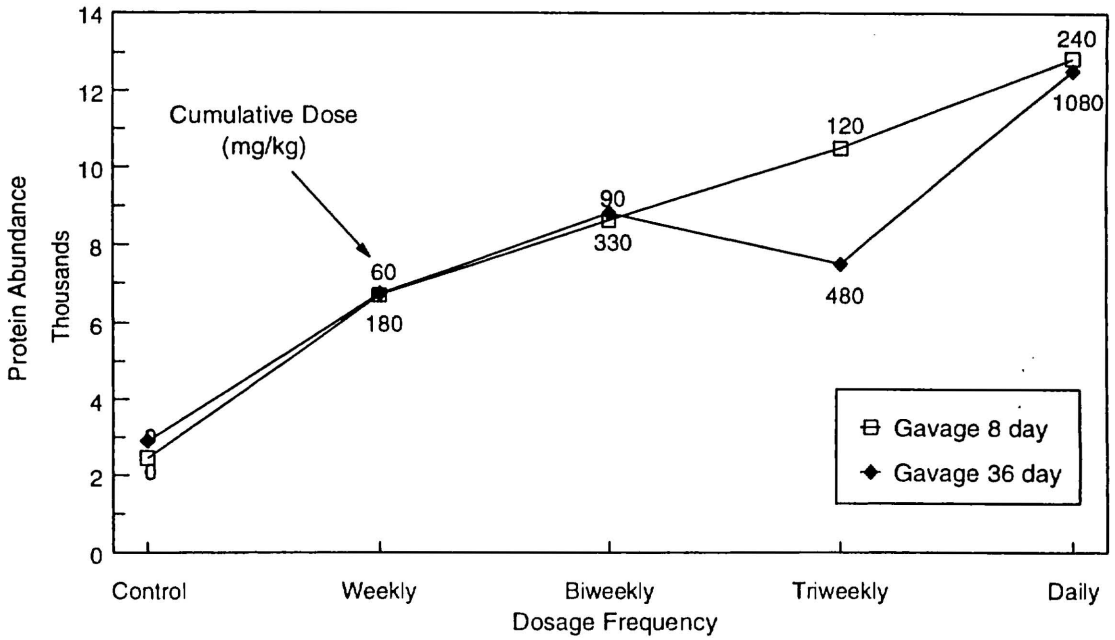


Fig. 4

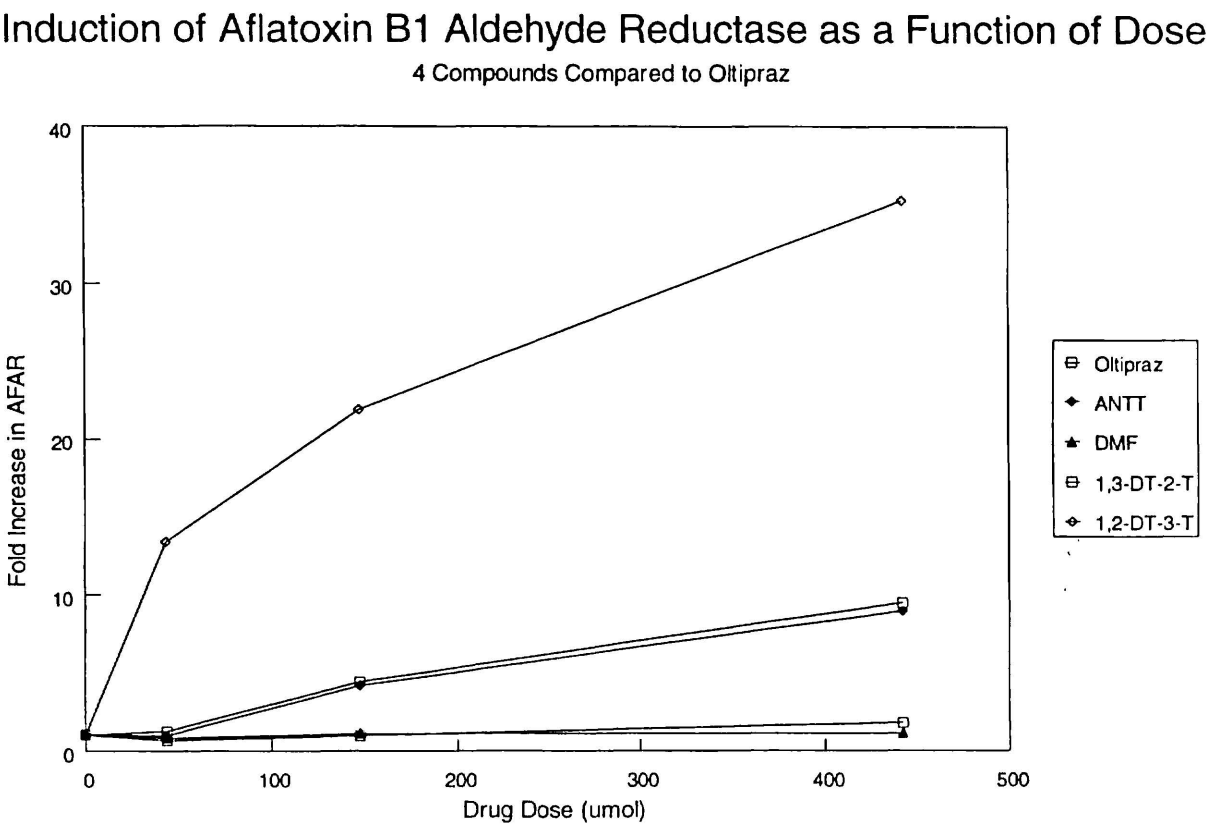
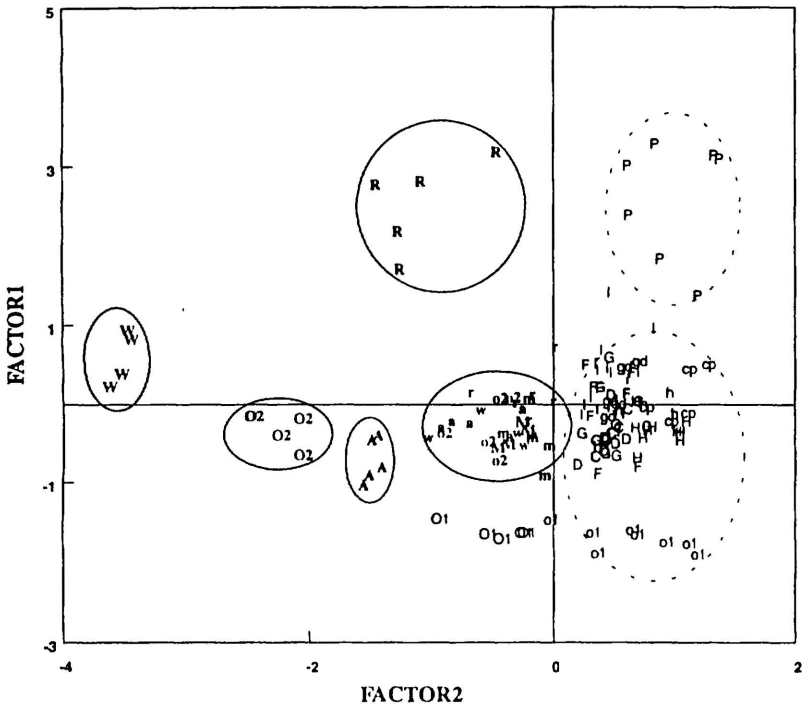

Induction of Aflatoxin B1 Aldehyde Reductase as a Function of Dose
4 Compounds Compared to Oltipraz
Fold Increase in AFAR
40
30
20
10
0
0
100
200
300
400
500
Drug Dose (umol)
Oltipraz
ANTT
DMF
1,3-DT-2-T
1,2-DT-3-T

Fig. 5

Effects of Chemoprevention Compounds in Rat Liver

Principal Components Analysis of Data on 81 Spots



o1 Group 1: Oltipraz 21 days in feed + 2 days recovery controls
i Group 2: Ibuprofen 21day feed control
f Group 3: DFMO 21day feed control
h Group 4: 4-HPR 21day feed control
cp Group 5: Carbenoxolone and Piroxicam 21day feed control
gd Group 6: Ca glutarate 21day and DHEA analogue 21day feed control
O1 Group 7: Oltipraz 21 days in feed + 2 days recovery 100 mg/kg
I Group 8: Ibuprofen 21day feed MTD
F Group 9: DFMO 21day feed MTD
H Group 10: 4-HPR 21day feed MTD
C Group 11: Carbenoxolone 21 day feed MTD
P Group 12: Piroxicam 21 day feed MTD
G Group 13: Ca glutarate 21day feed MTD
D Group 14: DHEA analogue 21day feed MTD
o2 Group 15: Oltipraz (control)
O2 Group 16: Oltipraz (442umol)
a Group 17: ANTT (control)
A Group 18: ANTT (442umol)
m Group 19: DMF (control)
M Group 20: DMF (442umol)
r Group 21: 1,3-DT-2-T (control)
R Group 22: 1,3-DT-2-T (442umol)
w Group 23: 1,2-DT-3T (control)
W Group 24: 1,2-DT-3-T (442umol)

Fig. 6

TABLE I. Relationships Between Sets of Proteins Affected by Various Chemoprevention Agents

	Oltipraz	Piroxicam	4-HPR	Ibuprofen	Carben-oxolone	Ca-gluc-arate	DHEA analog	DFMO
Oltipraz	26	0	1	0	0	0	0	0
Piroxicam		34	1	0	1	0	0	0
4-HPR			14	0	0	0	0	0
Ibuprofen				1	0	0	0	0
Carbenoxolone					4	0	0	0
Ca-glucarate						1	0	0
DHEA analog							4	0
DFMO								0

Table 1