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Combination Chemoprevention of Rat Mammary Carcinogenesis by Indomethacin and Butylated Hydroxytoluene[1]

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ABSTRACT

Indomethacin, a nonsteroidal antiinflammatory agent which inhibits prostaglandin biosynthesis, is an effective inhibitor of mammary carcinogenesis in rats. However, the activity of indomethacin as a chemopreventive agent is limited by toxicity. The present studies were conducted to determine if the toxic and anticarcinogenic effects of indomethacin can be modified by the phenolic antioxidant, butylated hydroxytoluene (BHT). Simultaneous administration of BHT resulted in a dose-related inhibition of indomethacin toxicity in female Sprague-Dawley rats, and increased the tolerable indomethacin dose from 50 to 150 mg/kg diet. When BHT (5000 mg/kg diet) and indomethacin (50 mg/kg diet) were administered in combination, no increased inhibition of 7,12-dimethylbenz(*a*)anthracene-induced mammary carcinogenesis was observed above that attained by administration of BHT alone or indomethacin alone at those doses. However, when the indomethacin dose was increased to 100 mg/kg diet, an enhanced inhibition of carcinogenesis was attained when BHT and indomethacin were administered from 2 weeks prior to until 1 week after 7,12-dimethylbenz(*a*)anthracene administration. These data indicate that "combination chemoprevention" regimens can be utilized to reduce the toxicity of anticarcinogenic drugs. However, the BHT-indomethacin interaction appears to involve a functional or dispositional antagonism which limits the anticarcinogenic efficacy of increasing indomethacin dose level.

INTRODUCTION

The inhibition of carcinogenesis through administration of pharmacological agents during the preneoplastic period has been termed "chemoprevention" (2). A wide variety of agents, with diverse chemical structures and putative mechanisms of action, have significant chemopreventive activity in animal models for human cancer (for reviews, see Refs. 3 and 4). Administration of chemopreventive compounds to carcinogen-treated animals results in a decrease in cancer incidence or multiplicity, and/or an increase in tumor latent period, in comparison to control animals not exposed to the chemopreventive agent. The efficacy of chemoprevention as a strategy for cancer control in humans will soon be examined in clinical trials (5).

Although significant reductions in carcinoma incidence and multiplicity have been achieved with the experimental use of chemopreventive drugs, the anticarcinogenic activity of compounds presently available is incomplete: cancer incidence is not reduced to zero with administration of these agents at nontoxic levels. The toxicity of anticarcinogenic agents remains the single most important factor limiting their activity. If this toxicity can be reduced, higher doses of the compounds could be administered without adverse effect, presenting the possibility of greater anticarcinogenic efficacy.

Two distinct approaches have been taken in order to develop chemopreventive regimens with increased activity and reduced toxicity. The first has involved the design of synthetic congeners of active compounds, in the attempt to dissociate chemopreventive activity from toxicity. This approach has been used most extensively with retinoids: synthetic retinoids have been designed which are less toxic than natural vitamin A compounds, yet which have equal or greater anticarcinogenic activity (3, 6, 7). The second means by which the efficacy of cancer chemoprevention may be increased is to administer anticarcinogenic agents simultaneously with other compounds. Such "combination chemoprevention" protocols seek to increase the efficacy of cancer prevention either through an additive or synergistic interaction between two chemopreventive agents (8, 9), or through the reduction of agent toxicity, thereby permitting administration of an active compound at higher dose levels.

We have previously reported that the phenolic antioxidant, BHT,[3] and the nonsteroidal antiinflammatory agent, indomethacin, are both effective inhibitors of mammary carcinogenesis induced in rats by DMBA. Reductions in mammary tumor multiplicity were observed when BHT and indomethacin were administered as single agents either for a short period around the time of carcinogen exposure, or chronically following clearance of the DMBA from the mammary gland (10, 11). The present experiment was conducted to determine if the efficacy of chemoprevention observed when these two compounds are administered as single agents is increased when they are administered in combination.

MATERIALS AND METHODS

Virgin, female Sprague-Dawley [Hsd:(SD)BR] rats were obtained as weanlings from Harlan/Sprague-Dawley, Indianapolis, IN. Animals were housed three to a cage in a room maintained at 22 ± 1°C on a 14-h light, 10-h dark cycle. All animals were allowed free access to drinking water and diet throughout the studies, except for a 16-h period prior to carcinogen administration; during this period, rats had access to drinking water only. Basal diet for the studies was Wayne Laboratory Chow (Allied Mills, Chicago, IL). Basal diet was supplemented with indomethacin (Sigma Chemical Co., St. Louis, MO) or BHT (Sigma) as required by the protocols. Indomethacin and BHT were administered in a quantity of sucrose carrier such that the addition of the chemopreventive agent plus carrier totalled 10 g/kg diet. Control diet contained sucrose carrier only. All food and bedding materials were changed twice weekly.

Toxicology Studies. On the basis of a previous report that the gastrointestinal toxicity of indomethacin could be modified by concomitant exposure to antioxidants (12), a pilot study was conducted to determine the effects of simultaneous exposure to BHT or BHA on rats fed a toxic dose of indomethacin. Data from this study (not shown) demonstrated that the induction of intestinal adhesions by indomethacin administered at a level of 100 mg/kg diet could be prevented by concomitant exposure to 2500 mg BHT/kg diet; by contrast, exposure to the same level of BHA exacerbated indomethacin toxicity.

In order to investigate further the interactions between BHT and indomethacin, an experiment was conducted to examine the dose

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[3] The abbreviations used are: BHT, 3,5-di-*tert*-butyl-4-hydroxytoluene (butylated hydroxytoluene); DMBA, 7,12-dimethylbenz(*a*)anthracene; BHA, 2(3)-*tert*-butyl-4-hydroxyanisole(butylated hydroxyanisole).

response effects of BHT protection on indomethacin toxicity. In this study, groups of 10 rats were fed indomethacin at levels of 75, 100, 125, or 150 mg per kg diet, with BHT added at levels of 0, 1000, 3000, or 5000 mg per kg diet. Control rats were fed indomethacin at 50 mg per kg diet, a level previously determined to be tolerable to Sprague-Dawley rats (11). Animals were observed daily for indications of indomethacin toxicity, and were weighed weekly. At necropsy, the presence or absence of gross intestinal toxicity was confirmed, and tissue samples were taken for histopathological evaluation. Tolerable dietary levels of indomethacin were defined as those which induced no mortality and no suppression of body weight gain in comparison to control, and which induced no grossly visible erosions or adhesions in the gastrointestinal tract.

Carcinogenesis Study. At age 36 days, rats were randomized by weight into groups of 25 according to the protocol (Table 1). With day of DMBA administration defined as time 0, groups of rats received indomethacin and/or BHT supplements from either 2 weeks prior to until 1 week after DMBA administration (−2 to +1 week), or beginning 1 week post-DMBA and continuing until the end of the experiment (+1 week to end). When not receiving a diet supplemented with indomethacin and/or BHT, animals were fed basal diet containing added sucrose carrier only.

At age 50 days, animals received a single intragastric instillation of 10 mg DMBA dissolved in 1.0 ml sesame oil. Beginning 4 weeks after DMBA administration, rats were palpated twice weekly to monitor mammary tumor appearance. Rats were weighed weekly, and observed twice daily for any signs of chemopreventive agent toxicity. Moribund animals were killed; otherwise, all rats were killed via CO_2 asphyxiation at 190 days after DMBA administration. Animals killed or found dead were necropsied promptly; mammary tumors were removed and coded by location, and any other abnormal tissues were removed for histological study. Tissues were fixed in 10% buffered formalin, stained with hematoxylin and eosin, and were classified histopathologically. Mammary tumor pathology was defined according to the criteria of Young and Hallowes (13). Only histologically confirmed mammary tumors were used in the data analysis.

Values for mammary tumor incidence and multiplicity were calculated by the life table method (14); tumor response data thus includes adjustments for intercurrent mortality. Tumor multiplicity calculations were based on the total number of rats at risk at each time point, and as such are not limited to tumor-bearing rats only. Comparisons of terminal cancer incidence and animal survival were made via χ^2 analysis; intergroup comparisons of tumor multiplicity were performed via analysis of variance, using square root transformed data, as suggested by Snedecor and Cochran (15). Values for the median tumor induction time were compared using the median test (16). Comparisons of mean group body weight were made by analysis of variance.

RESULTS

Toxicology Study. Simultaneous administration of BHT resulted in a significant inhibition of indomethacin toxicity; the efficacy of BHT protection was a function of both BHT dose and indomethacin dose. As indicated in Fig. 1*A*, BHT doses of 1000, 3000, and 5000 mg/kg diet were equally effective in protecting against toxicity induced by 75 mg indomethacin/kg diet: in all cases, concomitant administration of BHT resulted in complete protection against indomethacin toxicity over the course of a 90-day experiment. However, at higher indomethacin doses, a differential effect of the three BHT doses was observed. At a level of 100 mg indomethacin/kg diet, the 1000-mg BHT dose reduced the incidence of indomethacin toxicity, but did not prevent its occurrence (Fig. 1*B*). Increasing the indomethacin dose through 125 to 150 mg/kg diet resulted in a total loss of protection afforded by the 1000 mg BHT/kg diet dose, although rats fed BHT at levels of 3000 and 5000 mg/kg diet remained completely protected (Fig. 1, *C* and *D*). Animals fed BHT at 3000 or 5000 mg/kg diet showed no mortality or suppression of body weight gain in comparison to controls, regardless of the level of the dietary indomethacin supplement.

Carcinogenesis Study. The effects of nontoxic levels of indomethacin and/or BHT on mammary carcinogenesis induced by DMBA are summarized in Table 1. Confirming our previous report (10), administration of BHT at a level of 5000 mg/kg diet had significant chemopreventive activity when the supplement was given either from weeks −2 to +1, or from week +1 until the end of the experiment. Administration of BHT by the −2- to +1-week protocol (group 3) resulted in a statistically significant reduction in mammary cancer incidence; this chemoprevention protocol also reduced mammary cancer multiplicity by 60% and total mammary tumor multiplicity by approximately 40% from levels seen in the control group ($P < 0.01$). Administration of BHT by the +1-week to end schedule (group 7) reduced tumor multiplicity by approximately 30% in comparison to control ($P < 0.05$); consistent with our earlier findings (10), however, most of the reduction in mammary tumor multiplicity achieved by postcarcinogen administration of BHT came from a reduction in the number of benign mammary tumors, rather than through a decrease in carcinoma multiplicity.

Indomethacin administered at a level of 50 mg per kg diet was also effective in the inhibition of mammary carcinogenesis induced by DMBA. Chronic, postcarcinogen exposure to indomethacin (group 6) reduced mammary carcinoma multiplicity by one-fourth ($P < 0.05$) and total tumor multiplicity by more than one-third ($P < 0.01$) in comparison to dietary controls. However, the approximately 20% reduction in mammary tumor number resulting from administration of indo-

Table 1 *Influence of BHT and indomethacin on mammary tumor induction in rats treated with 10 mg DMBA*

Group	BHT dose (mg/kg diet)	Indomethacin dose (mg/kg diet)	Modifier administration protocol (wk)	Survival (%)	Cancer incidence (%)	Cancers/rat	Benign tumors/rat	Total tumors/rat	Body wt (g)
1	0	0		80	92	3.46	3.65	7.11	281 ± 5[a]
2	0	50	−2 to +1	72	95	3.33	2.47[b]	5.80	291 ± 7
3	5000	0	−2 to +1	88	68[b]	1.40[c]	2.67[b]	4.07[c]	278 ± 5
4	5000	50	−2 to +1	76	61[b]	1.98[c]	1.99[c]	3.97[c]	276 ± 5
5	5000	100	−2 to +1	100[d]	72	1.14[c]	2.02[c]	3.16[c]	274 ± 4
6	0	50	+1 to end	80	81	2.56[b]	1.88[c]	4.44[c]	279 ± 6
7	5000	0	+1 to end	84	96	3.33	1.71[c]	5.04[b]	305 ± 8[c]
8	5000	50	+1 to end	92	76	2.98	1.65[c]	4.63[c]	291 ± 4
9	5000	100	+1 to end	80	82	2.86	1.66[c]	4.52[c]	281 ± 7

[a] Mean ± SE.
[b] $P < 0.05$ *versus* group 6.
[c] $P < 0.01$ *versus* group 6.
[d] $P < 0.06$ *versus* group 6.

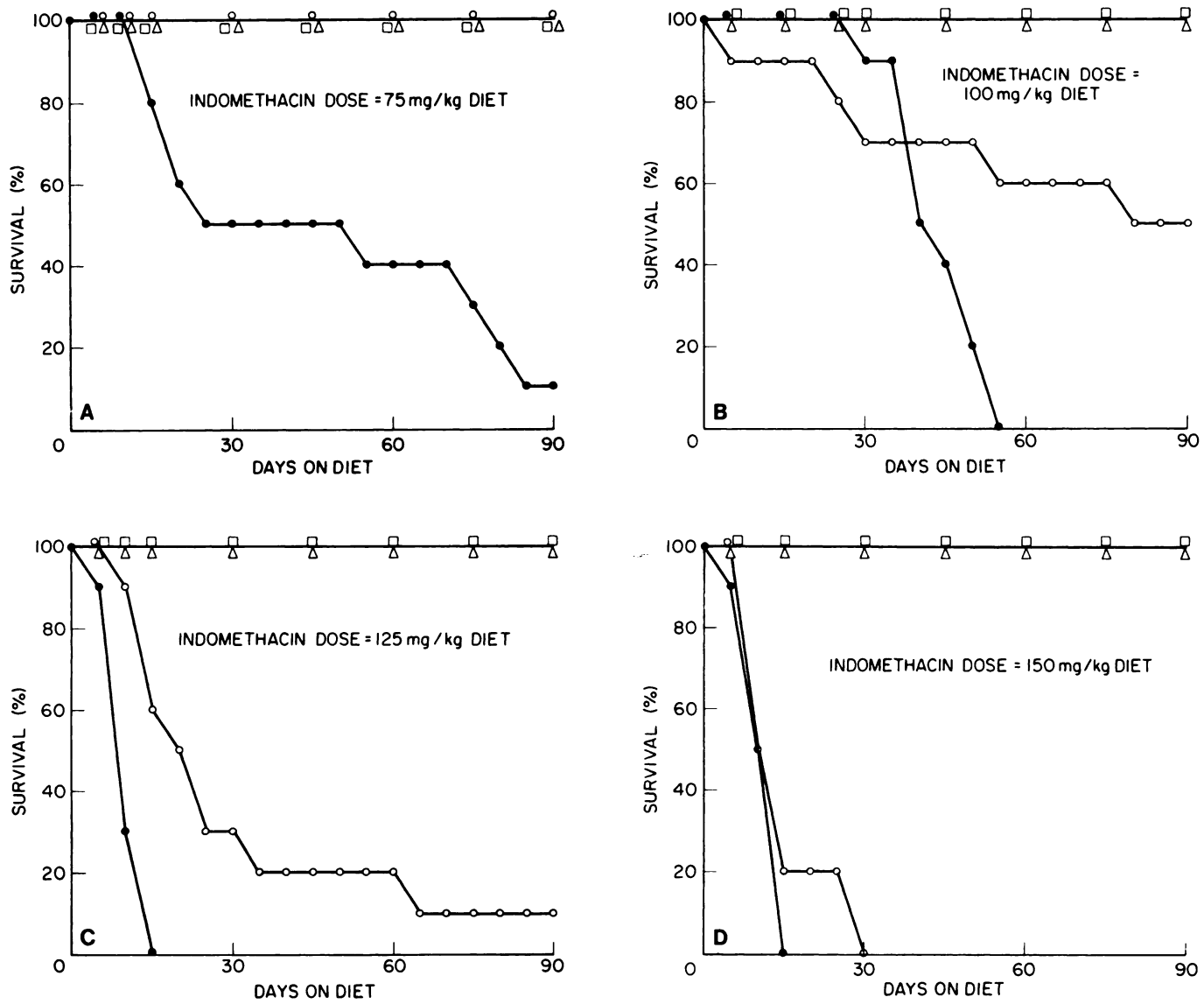

Fig. 1. Effect of BHT dose on survival in rats fed indomethacin. *A*, indomethacin, 75 mg/kg diet; *B*, indomethacin, 100 mg/kg diet; *C*, indomethacin, 125 mg/kg diet; and *D*, indomethacin, 150 mg/kg diet. ●, control (no BHT); ○, BHT (1000 mg/kg diet); □, BHT (3000 mg/kg diet); △, BHT (5000 mg/kg diet).

methacin by the −2- to +1-week protocol (group 2) was not significant at the 5% level.

Administration of indomethacin at a dose of 50 mg/kg diet in combination with BHT at 5000 mg/kg diet was also effective in mammary cancer chemoprevention, although the anticarcinogenic activity of the combined regimen was not significantly greater than that achieved by indomethacin alone or BHT alone. Administration of 50 mg indomethacin plus 5000 mg BHT by the −2- to +1-week protocol (group 4) reduced cancer incidence to 61% from 92% in the dietary control group, and reduced mammary tumor multiplicity by 45% compared to controls. While this inhibition is highly significant ($P < 0.01$), the activity of the combined regimen administered by the −2- to +1-week protocol is not greater than the inhibition achieved by administration of BHT alone by this schedule (group 3). Similarly, chronic, postcarcinogen administration of 50 mg indomethacin plus 5000 mg BHT/kg diet (group 8) reduced total tumor multiplicity by one-third ($P < 0.01$). However, the inhibition of carcinogenesis achieved by exposure to BHT plus indomethacin from week +1 until the end of the experiment was somewhat less than that obtained with administration of indomethacin alone by this protocol (group 6).

Whereas no enhancement of chemopreventive activity was observed when 5000 mg BHT was administered in combination with the 50 mg/kg diet dose level of indomethacin, an increase in anticarcinogenic efficacy was observed when this dose of BHT was combined with indomethacin at 100 mg/kg diet. As indicated above, administration of indomethacin at 100 mg/kg diet without simultaneous exposure to BHT induces lethal toxicity in rats within 10 to 15 days. However, concomitant administration of BHT with this dose of indomethacin not only protected against the induction of toxicity, but also increased anticarcinogenic activity in the −2- to +1-week protocol above that seen with tolerable levels of either agent alone (Fig. 2).

As defined by percentage of reduction in mammary carcinoma multiplicity and effects on animal survival, administration of 100 mg indomethacin plus 5000 mg BHT/kg diet from weeks −2 to +1 (group 5) was the most effective chemopreventive regimen in the present study (Table 1). This protocol reduced carcinoma multiplicity by more than 65%, and decreased total tumor number by more than 55% in comparison to control ($P < 0.01$); furthermore, this was the only chemoprevention regimen to cause a statistically significant reduction in tumor-related mortality in the present study. Administration

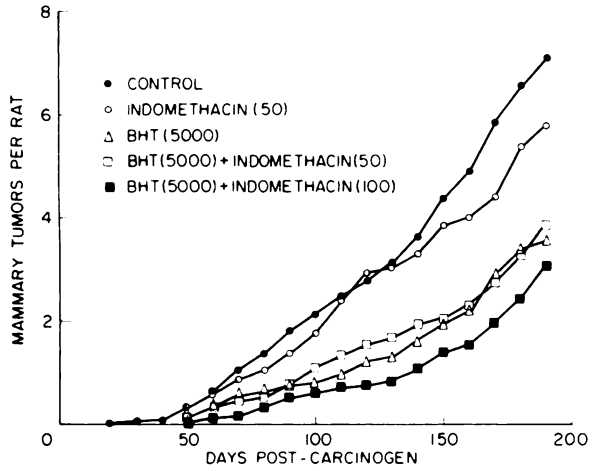

Fig. 2. Effect of BHT and indomethacin administered from weeks −2 to +1 on mammary tumor multiplicity. *Numbers in parentheses*, mg/kg diet.

of BHT in combination with the high dose of indomethacin also increased the median tumor induction time from 78 days in the control group to 131 days ($P < 0.06$). By contrast, administration of BHT in combination with high dose of indomethacin from week +1 until the end of the study (group 9) was no more effective in mammary cancer chemoprevention than were BHT alone, indomethacin alone, or the combination of BHT plus the lower dose of indomethacin (groups 6, 7, and 8, respectively).

DISCUSSION

Although previous studies conducted in several laboratories have demonstrated the efficacy of systemic administration of indomethacin in the prevention of cancer induction in the mammary glands (11, 17), colon (18, 19), and esophagus (20) of experimental animals, the dose levels required for effective chemoprevention by indomethacin are close to the threshold of lethal toxicity. The toxic:effective dose ratio of indomethacin for modulation of mammary carcinogenesis in Sprague-Dawley rats is quite small: chemoprevention of mammary carcinogenesis induced by high doses of DMBA requires an indomethacin dose of approximately 50 mg/kg diet (11), while acute toxicity is induced in this strain at 75 mg indomethacin/kg diet. Thus, the toxic:effective dose ratio is approximately 1.5:1. In the present experiment, the administration of indomethacin in combination with 5000 mg BHT/kg diet provided a much larger increment between doses which are effective in chemoprevention and those which induce toxicity: while indomethacin administered at levels of 50 or 100 mg/kg diet in combination with BHT was effective in cancer prevention, no toxicity was observed when indomethacin doses of up to 150 mg/kg diet were administered with BHT. Thus, coadministration of BHT with indomethacin increases the toxic:effective dose ratio from approximately 1.5:1 to at least 3:1.

The dissociation of anticarcinogenic activity from toxicity is one goal of combination chemoprevention studies. In the present study, the reduction of indomethacin toxicity through coadministration of BHT resulted in at least a doubling of the ratio between toxic and effective doses, and provided a large increase in the "margin of safety" for the use of indomethacin as a chemopreventive agent in rats. However, a corollary goal of combination chemoprevention is to utilize any such reduction in toxicity to increase anticarcinogenic efficacy. In this regard, the combined chemoprevention regimen of BHT plus indomethacin was less successful.

While the 50 mg/kg diet dose of indomethacin and the 5000 mg/kg diet dose of BHT were both effective in preventing mammary tumor induction in the present study, no increase in anticarcinogenic activity was observed when these doses of indomethacin and BHT were administered in combination. At least two hypotheses could explain this lack of enhanced chemoprevention. It could be postulated that indomethacin and BHT act to inhibit mammary carcinogenesis through a common mechanism. Under such a circumstance, an increased inhibition of carcinogenesis would not be expected with simultaneous administration of the two agents. Alternatively, some functional or dispositional antagonism may exist between BHT and indomethacin; any such antagonism could limit the anticarcinogenic efficacy of the combined treatment protocol. In this regard, the data from the present study are similar to those of our previous report of the influence of *N*-(4-hydroxyphenyl)retinamide, a synthetic retinoid, and maleic anhydride-divinyl ether copolymer, an inducer of interferon biosynthesis, on mammary carcinogenesis (21). While both *N*-(4-hydroxyphenyl)retinamide and maleic anhydride-divinyl ether copolymer were active as single agents, no increase in anticarcinogenic efficacy was observed when they were administered in combination. This lack of interaction may be attributed to retinoid transcriptional control of interferon biosynthesis, and inhibition of its action, examples of functional antagonism which have been reported in the literature (22, 23).

A similar conclusion regarding antagonism between BHT and indomethacin can be reached through analysis of the tumor response observed in groups exposed to BHT in combination with the 100-mg dose of indomethacin: although no increase in anticarcinogenic activity was observed with administration of this combination by the +1-week to end protocol, an increase in chemopreventive efficacy did occur when the regimen was given by the −2- to +1-week schedule. This differential interaction between BHT and indomethacin in the −2- to +1-week *versus* the +1-week to end administration schedules suggests that the compounds interact differently to inhibit carcinogenesis in these two phases of mammary tumor induction, and thus argues against the common mechanism hypothesis.

Although an enhanced inhibition of carcinogenesis was found with administration of BHT in combination with the high dose of indomethacin from weeks −2 to +1, this increased anticarcinogenic activity was less than would be expected on the basis of an additive interaction between the two agents. Furthermore, no increase in anticarcinogenic activity was obtained when this regimen was administered to DMBA-treated rats beginning 1 week after the carcinogen. Thus, BHT protection against indomethacin toxicity is achieved at the cost of at least a partial loss of anticarcinogenic activity. The mechanism(s) by which BHT acts to modify indomethacin toxicity and chemopreventive activity is currently under investigation.

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