Correlation between Electron-donating Ability of a Series of 3-Nitroflavones and Their Efficacy to Inhibit the Onset and Progression of Aberrant Crypt Foci in the Rat Colon

Vernon E. Steele, Charles W. Boone, Daniel Dauzonne, Chinthalapally V. Rao, and René V. Bensasson

ABSTRACT

A series of five 3-nitroflavones were tested for their ability to inhibit the formation of colon aberrant crypt foci (ACF) induced by a s.c. injection of azoxymethane (C6H6N2O) in rats. Our aim was to relate the electron-donating effects of the 3-nitroflavones as characterized by their Hammett substitution constants with their efficacy in inhibiting ACF. In a first assay (initiation, protocol A) the 3-nitroflavones as well as the 4'-substituted nitro-, methoxy-, fluoro-, and hydroxy-3-nitroflavones were continuously present in the diet. In a second assay (postinitiation, protocol B) they were given for a period of 4 weeks after the last azoxymethane injection. The different substituents of the 3-nitroflavones at the 4'-position spanned a spectrum of Hammett constants (σp), going from +0.79 for the electron-withdrawing group, NO2, to −0.92 for the electron-donating group, OH. For both protocols the percentages of inhibition plotted versus the Hammett substitution constants showed a linear correlation, the most efficacious ACF inhibition being produced by the molecules with the most electron-donating substituents. Moreover, the nitroflavones were not only chemoprotective during initiation of the ACF, but also therapeutic in the postinitiation progression assay. The above correlations may be of predictive value in the search for new chemoprotective agents. The overall molecular mechanism of the inhibition of ACF by the 3-nitroflavones under study appears to involve redox reactions.

INTRODUCTION

A number of natural and synthetic compounds have been identified as potential agents against colon tumor development. Several epidemiological studies and clinical trials have demonstrated that treatment with NSAIDs4 caused regression of pre-existing colonic adenomas. The most active chemoprotective agents against colonic precancerous or cancerous lesions investigated in animal models include NSAIDs and retinoids (1), organoselenium compounds (2, 3), and flavonoids (4, 5). The presence of ACF in the colon mucosa of patients with colon cancer, the expression of mutations in APC and ras, and overexpression of cyclooxygenase-2 and inducible nitric oxide synthase in the aberrant crypts suggest that ACF are precursor lesions from which adenomas and carcinomas develop in the colon. Also, in animal model studies there have been a positive correlation between ACF formation and colon tumor incidence and multiplicity. In the present work, five systematically substituted 3-nitroflavones were tested for their ability to inhibit the formation of colon ACF. Our aim was to verify whether the efficacy of chemoprotection against

MATERIALS AND METHODS

Chemopreventive Agents and Carcinogen. The 3-nitroflavone and four analogues were synthesized on a multigram scale, using a procedure described previously (6, 7). The four analogues were substituted on the 4'-position of the B ring: 4'-OH, 4'-OCH3, 4'-F, and 4'-NO2 (Fig. 1).

The carcinogen AOM, purchased from Ash Stevens Inc. (Detroit, MI), was injected at a dose level of 15 mg/kg body weight. The negative control animals were injected with saline in place of AOM. The rats were sacrificed by CO2 asphyxiation and the colon removed for measuring ACF. This study was approved by the Institutional Animal Care and Use Committee at the American Health Foundation.

ACF Assay. The in vivo chemoprotective activity of the five 3-nitroflavones was measured using a rat colon ACF assay, described earlier by Wargovich et al. (1). The timeline of this assay is shown in Fig. 2. In brief, F344 rats, purchased from Charles River Breeding Laboratories (Kingston, NY) were randomized into groups of 10 at 7 weeks of age. All of the rats were fed the AIN-76A diet (Dyets Inc., Bethlehem, PA) for the duration of the experiment. All of the rats were injected at 8 and 9 weeks of age with the carcinogen AOM (15 mg/kg body weight) to induce aberrant colonic crypts. Two protocols were used to determine the effects of the 3-nitroflavonon ACF formation (Fig. 2). Protocol A was designed to measure the ability of 3-nitroflavones to block the onset of ACF formation induced by AOM. Protocol B was designed to evaluate the efficacy of 3-nitroflavones to prevent ACF progression after it had been initiated. Under the initiation protocol A, the animals were fed from 7–13 weeks of age with one of the following dose regimens of 3-nitroflavones: 63 ppm, 125 ppm, or no test compound, and sacrificed at the end of 13 weeks of age. Under the postinitiation protocol B, the animals were exposed to carcinogen as in protocol A at week 8 and 9, but were fed the test diets at the same two concentrations between 12 and 16 weeks of age, and sacrificed at the end of 16 weeks of age. In both protocols, the colon were removed and fixed in cold PBS, cut longitudinally, and stained with 0.2% methylene blue (8). The aberrant crypts of the entire colon were scored under a dissecting microscope at ×40 magnification, and the mean number of aberrant crypts per colon determined. The aberrant crypt multiplicities (crypts/colon) at both treatment doses were statistically compared with the carcinogen-only group using the unpaired t test and were based on “Bonferroni’s Correction,” an adjustment of P in multiple comparisons with a single control group. Statistical significance was set at P < 0.05. Correlation coefficients were determined by the least squares method.
period when 3-nitroflavone derivative was present in the diet. Electron-donating substituents, 4 significant inhibition of ACF onset and progression was observed for the ppm. Neither dose caused measurable weight changes or other toxic comparative purposes: a low dose of 63 ppm and a high dose of 125 ppm. The effects of the 3-nitroflavones on AOM-induced ACF formation in rat colon for protocol A and protocol B are reported in Tables 1 and 40% inhibition for protocols A and B, respectively, as compared with the other three cases. For the other three assays, corresponding plots for the percentage of inhibition of ACF versus the Hammett constant. For protocol A, where the 3-nitroflavone chemoprotective agent is present at the onset of ACF formation, the correlation coefficients are 0.95 and 0.91 for the 63 and 125 ppm doses, respectively. Under protocol B, where the 3-nitroflavones were administered during the progression phase of the ACFs, the correlation coefficients are 0.88 and 0.89 for the 63 and 125 ppm dose groups, respectively. For protocol A at the 63 ppm dose, the order of efficacy is OH > OCH3 > F > H > NO2 corresponding exactly to the order of the Hammett constants, and the correlation coefficient is the highest as compared with the other three cases. For the other three assays, Table 1 Mean value of colon ACF per rat measured after the initiation phase (protocol A; n = 10)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration of 3-nitroflavones in diet</th>
<th>Total ACF/rat ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>63 ppm</td>
<td>125 ppm</td>
</tr>
<tr>
<td>Control (no nitroflavone)</td>
<td>94 ± 8.7</td>
<td>94 ± 8.7</td>
</tr>
<tr>
<td>3-4'-Dinitroflavone</td>
<td>96 ± 10</td>
<td>88 ± 9.3</td>
</tr>
<tr>
<td>3-Nitroflavone</td>
<td>80 ± 8.2</td>
<td>73 ± 7.9</td>
</tr>
<tr>
<td>4'-Fluoro-3-nitroflavone</td>
<td>72 ± 7.3</td>
<td>58 ± 6.2</td>
</tr>
<tr>
<td>4'-Methoxy-3-nitroflavone</td>
<td>70 ± 7.5</td>
<td>59 ± 6.8</td>
</tr>
<tr>
<td>4'-Hydroxy-3-nitroflavone</td>
<td>63 ± 7.0</td>
<td>51 ± 6.1</td>
</tr>
</tbody>
</table>

a Significant inhibition at P < 0.01–0.001.  
b Significant inhibition at P < 0.05.

Table 2 Mean value of colon ACF measured after the progression phase (protocol B; n = 10)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration of 3-nitroflavones in diet</th>
<th>Total ACF/rat ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>63 ppm</td>
<td>125 ppm</td>
</tr>
<tr>
<td>Control (no nitroflavone)</td>
<td>132 ± 11</td>
<td>132 ± 11</td>
</tr>
<tr>
<td>3-4'-Dinitroflavone</td>
<td>130 ± 13</td>
<td>131 ± 12</td>
</tr>
<tr>
<td>3-Nitroflavone</td>
<td>126 ± 12</td>
<td>112 ± 11</td>
</tr>
<tr>
<td>4'-Fluoro-3-nitroflavone</td>
<td>103 ± 10</td>
<td>83 ± 8.8</td>
</tr>
<tr>
<td>4'-Methoxy-3-nitroflavone</td>
<td>96 ± 9.4 b</td>
<td>79 ± 7.1 b</td>
</tr>
<tr>
<td>4'-Hydroxy-3-nitroflavone</td>
<td>99 ± 10</td>
<td>80 ± 7.8 a</td>
</tr>
</tbody>
</table>

a Significant inhibition at P < 0.01–0.001.  
b Significant inhibition at P < 0.05.

Table 3 The percentage of inhibition of colon ACF by low-dose 3-nitroflavones under protocol A compared with their Hammett constants

<table>
<thead>
<tr>
<th>% Inhibition (±SE)</th>
<th>Concentration in diet</th>
<th>Hammett constant σ&lt;sub&gt;H&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>63 ppm</td>
<td>125 ppm</td>
</tr>
<tr>
<td>3-4'-Dinitroflavone</td>
<td>−2.1 ± 13.3</td>
<td>6.4 ± 12.7</td>
</tr>
<tr>
<td>3-Nitroflavone</td>
<td>14.8 ± 12.0</td>
<td>22.3 ± 11.8</td>
</tr>
<tr>
<td>4'-Fluoro-3-nitroflavone</td>
<td>23.4 ± 11.4</td>
<td>38.3 ± 10.7</td>
</tr>
<tr>
<td>4'-Methoxy-3-nitroflavone</td>
<td>25.5 ± 11.5</td>
<td>37.2 ± 11.0</td>
</tr>
<tr>
<td>4'-Hydroxy-3-nitroflavone</td>
<td>32.9 ± 11.2</td>
<td>45.7 ± 10.6</td>
</tr>
</tbody>
</table>

Table 4 The percentage of inhibition of colon ACF by high-dose 3-nitroflavones under protocol B compared with their Hammett constants

<table>
<thead>
<tr>
<th>% Inhibition (±SE)</th>
<th>Concentration in diet</th>
<th>Hammett constant σ&lt;sub&gt;H&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>63 ppm</td>
<td>125 ppm</td>
</tr>
<tr>
<td>3-4'-Dinitroflavone</td>
<td>1.5 ± 16.9</td>
<td>0.8 ± 16.3</td>
</tr>
<tr>
<td>3-Nitroflavone</td>
<td>4.6 ± 16.6</td>
<td>15.2 ± 15.7</td>
</tr>
<tr>
<td>4'-Fluoro-3-nitroflavone</td>
<td>21.9 ± 14.9</td>
<td>37.1 ± 14.4</td>
</tr>
<tr>
<td>4'-Methoxy-3-nitroflavone</td>
<td>27.3 ± 14.8</td>
<td>40.2 ± 13.4</td>
</tr>
<tr>
<td>4'-Hydroxy-3-nitroflavone</td>
<td>25.0 ± 15.4</td>
<td>39.4 ± 13.8</td>
</tr>
</tbody>
</table>
these orders are slightly different. These differences decrease the
correlation coefficients to a minor extent and are attributable to
slightly larger experimental errors for the three other assays. We have
used the $\sigma_p^+$ constants reported by Hansch and Leo (9). These
$\sigma_p^+$ constants, formulated for substituents capable of delocalizing a pos-
tive charge, give the best correlations for our data. It can be con-
cluded that the most efficacious derivatives have the best electron-
donating substituents.

**DISCUSSION**

Our hypothesis, that the 3-nitroflavone efficacy in inhibiting ACF
formation *in vivo* would be related to the increasing ability of these
different substituents to donate electrons, is confirmed by the high
linear correlation between the *in vivo* inhibition efficacy of the dif-
ferent 3-nitroflavones and their associated Hammett constants, which
characterize the ability to donate electrons (9–11). The regression
lines for data of protocols A and B give four equations (correlation
coefficients):

Protocol A (63 ppm): % of ACF onset $= 15.2 - 18.3 \sigma_p^+$
($r = 0.95$)

Protocol A (125 ppm): % of ACF onset $= 25.9 - 20.7 \sigma_p^+$
($r = 0.91$)

Protocol B (63 ppm): % of ACF progression $= 13 - 15.3 \sigma_p^+$
($r = 0.88$)

Protocol B (125 ppm): % of ACF progression $= 22.0 - 23 \sigma_p^+$
($r = 0.98$)

From these equations it is possible to predict that for better electron-
donating substituents such as $-N(CH_3)_2$ or $-N(CH_2 CH_3)_2$,
which have more negative Hammett constants (−1.70 and −2.07,
respectively) the percentage of inhibition might reach 60–70% in the
case of the most efficacious 125 ppm regimen.

A comparison between the chemoprotection by the 3-nitroflavones
in the initiation protocol A and that observed in the postinitiation
protocol B (Tables 3 and 4) shows that the most active molecules are
the same in both protocols and correspond to those with the most
negative Hammett constants relating directly to the best electron-
donating substituents. Moreover, the similar degree of inhibition
reached in both protocols A and B underscores the therapeutic effect
as well as the protective effect of these agents.

It is important to link our correlations for ACF inhibition efficacy
with similar linear correlations found previously for other redox-
related chemoprotection studies. These earlier correlations showed
that more easily oxidized agents are more efficacious. The agents
studied include: (a) a series of phenols able to inhibit benzo(a)pyrene-
induced neoplasia in the forestomach of mice (12); (b) a series of diphenols that induce production of cancer protective enzymes (13); and (c) a series of NSAIDs acting in vitro and in vivo (14–16). It is important to emphasize that NSAIDs show promise in the chemoprevention of cancer (17–19).

Such correlations dealing with chemoprotection redox mechanisms may be related to the redox-sensing of gene transcription (20–22). Moreover, metabolism of xenobiotics is often ruled by redox mechanisms, as shown by the following: (a) by the correlation of the rates of reduction of 27 molecules by purified cytochrome P-450 reductase with the one-electron reduction potentials of these compounds (23); or (b) by the demonstration that cytochrome P-450 mediates covariant binding of substrates to DNA via one-electron oxidation (24).

In conclusion, the correlations reported in the present work should help the search for and identification of more efficient chemoprotectors. Moreover, our present correlations underscore the link between redox mechanisms of ACF inhibition in the colon with the redox correlations observed in: (a) the chemoprotection by phenols against benzo(a)pyrene-induced neoplasia in the forestomach of mice (12); (b) in the activation by diphenols of chemoprotective enzyme synthesis (13); and (c) in the anti-inflammatory process (14–16). The latter observations suggest that in our case the mechanisms of retardation might involve enhancing of multiple detoxification systems as well as reduction of AOM-induced inflammation. Flavones are known to inhibit the inflammatory process (25, 26). Thus, the electron-donating molecules such as these nitroflavones may slow or block the progression phase (protocol B). For a better understanding of the molecular mechanisms of cancer chemoprotection, physicochemical properties of the many compounds already identified as efficient agents (27, 28) should be ranked versus physicochemical parameters, which include their redox properties. Such ranking would allow us to predict the cancer protective efficacy of certain classes of chemical agents and to approach some biochemical mechanisms of carcinogenesis and its chemoprotection.

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