Use of a Mammalian Cell Culture Benzo(a)pyrene Metabolism Assay for the Detection of Potential Anticarcinogens from Natural Products: Inhibition of Metabolism by Biochanin A, an Isoflavone from Trifolium pratense L

John M. Cassady, Thomas M. Zennie, Young-Heum Chae, Mark A. Feria, Nuris E. Portuondo, and William M. Baird

ABSTRACT

Based on the epidemiological evidence for a relationship between consumption of certain foods and decreased cancer incidence in humans, an assay was developed to screen and fractionate plant extracts for chemopreventive potential. This assay measures effects on the metabolism of [3H]benzo(a)pyrene (B(a)P) in hamster embryo cell cultures. Screening of several plant extracts has generated a number of activity leads. The 95% ethyl alcohol extract of one of these actives, Trifolium pratense L. Leguminosae, red clover, significantly inhibited the metabolism of [3H]B(a)P by 54% in comparison to control cultures and decreased B(a)P-DNA binding by 37 to 50% at a dose of 25 μg/ml. These studies demonstrate that the hydrocarbon metabolism assay can detect and guide the fractionation of potential anticarcinogens from plants. The ability of the isoflavone biochanin A to inhibit carcinogen activation in cells in culture suggests that in vivo studies of this compound as a potential chemopreventive agent are warranted.

INTRODUCTION

Humans are exposed to numerous carcinogens and mutagens daily, some avoidable (such as cigarette smoking) and some virtually unavoidable (diet, environmental pollution, oxygen radicals). The diet has been shown to have a profound effect on the incidence and location of various human cancers worldwide (1, 2), and epidemiological studies suggest that certain dietary components may help to prevent cancer induction. This prophylaxis has been termed cancer chemoprevention. Wattenberg (3) has demonstrated that such agents may inhibit cancer induction by a number of mechanisms. One of the more common mechanisms is through inducing alterations in the enzymatic activation or detoxification of carcinogens.

Although many biological assays have been used to examine the chemopreventive potential of various chemicals, there have been relatively few studies using activity-directed fractionation to isolate active compounds from plants. In addition, it is impractical to use in vivo models to guide these procedures. Loub et al. (4) used an activity-directed fractionation procedure based upon induction of aryl hydrocarbon hydroxylase activity in the liver and intestinal mucosa of Sprague-Dawley rats to isolate and identify several indoles from cruciferous vegetables. Kaweol and caffestol palmitates were isolated from green coffee beans (5) based upon an assay that measured the increase in glutathione S-transferase activity in liver and intestinal mucosa of mice. Practical assays for activity-directed fractionation of active plants must be rapid, sensitive, convenient, and capable of detecting alterations in carcinogen metabolism. In this paper, we describe the development and application of an assay that measures effects on the metabolism of [3H]B(a)P pyrene, a widespread environmental carcinogen, in early passage cultures of Syrian hamster embryo cells (6). The chemical and analytical procedures developed for activity-directed fractionation of antineoplastic compounds from plants (7, 8) were adapted to the isolation and identification of potential anticarcinogens from food and food plants, such as red clover extracts, which significantly inhibited the metabolism of benzo(a)pyrene and binding of B(a)P metabolites to DNA.

MATERIALS AND METHODS

Spectroscopy and Chromatography. 1H NMR in deuterchloroform was performed using a Varian XL-200, and 13C NMR in deuteriochloroform was measured on a Chemagnetics A-200 spectrometer. EI and CI mass spectra were obtained on a Finnigan 4700 quadrupole mass spectrometer. High-resolution mass spectra were recorded on a Kratos MS 50. The IR spectrum was performed on a Beckman IR-33 using a KBr pellet. UV spectra were measured on a Beckman DU-7 in methanol using sodium methoxide, AlCl3, HCl, and sodium acetate as UV shift reagents.

For flash column chromatography EM 9385 Silica Gel 60 was used for the adsorbent. Radial chromatography was performed on a Chromatotron Model 7924 using a 1-, 2-, or 4-mm rotor with EM 7749 Silica Gel 60 PF 254 as adsorbent. TLC plates were Merck 5714 Silica Gel 60 F254.

Cell Culture Toxicity Assay. Hamster embryo cell cultures were prepared and grown as described previously (6). Tertiary cultures were plated in 60-mm plastic dishes (Falcon) (5 x 105 cells), and 24 h later the test compound was added at 10-fold dilutions from 500 μg/ml of medium to 0.05 μg/ml for 24 h. At that time the cultures which were approximately 70% confluent were examined microscopically and subjectively evaluated for the percentage of the cells dividing and the cell density. The highest noninhibitory dose was selected for metabolism studies.

B(a)P Metabolism Assay. Tertiary hamster embryo cell cultures (106 cells per 25-cm2 flask, 3 flasks per group) were plated in 8 ml of medium containing 10% calf serum and refed with 8 ml of fresh medium after 48 h. Seventy-two h after plating, the cultures were treated with the test compound in DMSO or DMSO as a control, and 30 min later [3H]-B(a)P (1 μg/ml; specific radioactivity, 0.25 Ci/mmol) was added. Twenty-four h later medium was removed and stored at —20°C. Aliquots (0.2 ml) were extracted by a two-stage chloroform:methanol:water procedure (6, 9). The assay uses initial mixing with a vortex mixer in a single-phase system of chloroform:methanol:water (including the medium) (1:2:0.8) to ensure complete extraction of the lipophilic hydrocarbon and its metabolites following by addition of 1 ml of chloroform.

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was subjected to silica gel flash column chromatography with hexane, and 1 ml of water and mixing with a vortex mixer. After centrifugation for 10 min, the aqueous phase was removed and extracted with 2.0 ml of chloroform to ensure complete extraction. The chloroform extracts were then pooled, and the radioactivity in the organic and aqueous-methanol phases was measured by liquid scintillation counting of 0.1-
ml aliquots. This extraction procedure results in recovery of unmetabolized B(a)P and Phase I metabolites (dihydrodiols, quinones, and phenols) in the chloroform phase. The water-soluble metabolites including glucuronides and glutathione conjugates and multiple oxidation products are retained in the aqueous-methanol phase. Since the large majority of the metabolites formed from B(a)P in hamster embryo cells are water soluble (usually greater than 80%) (6), this assay provides a rapid measure of B(a)P metabolism.

BHA, a known inhibitor of carcinogenesis and B(a)P metabolism (10), was used to treat a positive control group in all assays at a concentration of 50 μg/ml of medium. The highest nontoxic dose of BHA was selected from multiple experiments using different hamster embryo cell preparations. Doses of 75, 65, 50, and 5 μg/ml of medium were tested, and the results show that 75 μg/ml were toxic and 65 μg/ml exhibited borderline toxicity, while 50 μg/ml showed a significant inhibition of B(a)P metabolism with no cell toxicity. The lowest dose, 5 μg/ml, produced no significant inhibition of B(a)P metabolism.

Using BHA as a positive control gave us an indication of the health and viability of the cells in the culture assay for that particular experiment and helped eliminate false negatives.

Analysis of B(a)P Metabolites. The B(a)P metabolites in the organic phase were analyzed by HPLC on an Ultrasphere C18 column (25 cm × 4.6 mm) eluted with a methanol:water gradient as described previously (6). UV absorbing standards of authentic B(a)P metabolites (Chemical Repository, Division of Cancer Etiology, National Cancer Institute) were included in each HPLC analysis. The radioactivity was monitored with a Flo-one β flow monitor set to update every 30 s.

Binding of B(a)P to DNA. Tertiary hamster embryo cell cultures (5 × 10⁶ cells) were plated in 175-cm² flasks containing 50 ml of minimal essential medium with 10% fetal bovine serum. After 2 days the cultures were refed with fresh medium and 24 h later with the test compound, or extract in DMSO was added. Five to 10 min later the cultures were treated with [3H]B(a)P (1 μg/ml of medium, 0.5 mCi/flask). After 24 h of incubation at 37°C the cells were harvested, and DNA was isolated as described previously (11). The radioactivity in an aliquot was measured by liquid scintillation counting, the amount of DNA was determined by A260, and these values were used to calculate the level of B(a)P metabolites bound to DNA.

After enzymatic degradation of the DNA to deoxyribonucleosides, the B(a)P:deoxyribonucleoside adducts were isolated by chromatography on Sep-Pak C18 cartridges and analyzed by HPLC on a 25-cm × 4.6-mm Ultrasphere C18 reversed-phase column (11). The column was eluted at a flow rate of 1.0 ml/min with methanol:water (46:54) for 34 min, a linear gradient for 10 min (46:54 to 55:45) and at 55:45 for 24 min. Fifteen 1.0-ml fractions followed by 165 fractions (0.3 ml) were analyzed by scintillation counting.

Plant Extraction. Leaves, stems, and flowers of Trifolium pratense L. (red clover) were collected. A voucher specimen is on deposit in the biology herbarium of the Department of Biology, Purdue University. The fresh plant (918 g) was ground with 2 liters of 95% ethyl alcohol in a commercial size Waring blender for 5 min. The blended material was then filtered through a Büchner funnel, and the filtrate concentrated in vacuo to give 46.5 g of the 95% ethyl alcohol extract. The 95% ethyl alcohol extract was then further partitioned according to the scheme shown in Fig. 1. Fifteen 1.0-ml fractions followed by 165 fractions (0.3 ml) were collected. These fractions were combined according to the fraction ID. This fraction was found to be active at twice the dose-response dose, led to the isolation of additional biochanin A, along with an analogue, formononetin (see Fig. 3). Formononetin was inactive in the metabolism bioassay. Biochanin A represented about 30% of the interface fraction.

RESULTS

The results of bioassay-directed fractionation of the active ethyl alcohol extract of red clover are presented in Fig. 1 and Table 1. The ethyl alcohol extract was active at doses from 500 μg/ml to 1000 μg/ml; however, toxicity was detected at the highest dose (see Table 1). Further partitioning of the active ethyl alcohol extract was dose responded from 750 μg/ml. After partitioning between chloroform and water, the activity appeared in the chloroform extract. Examination of the interface fraction which was active at twice the dose-response dose confirms the presence of biochanin A. Chromatography of the chloroform fraction gave active column Fraction 1D. This fraction was carried through two separations on the Chromatotron to give in turn active Fractions 2D an 3B. Crystallization of Fraction 3B gave 30 mg of the active constituent, biochanin A. Fractions 1A, 1B, 2B, and 2F show activity at twice the dose-response dose and are under further investigation. The B(a)P metabolites present in the organic phase of the sample treated with red clover extract at 300 μg/ml were analyzed by HPLC, and the amount of the major primary B(a)P metabolites was determined (Fig. 2). The two major changes were a slight increase in the amount of 9-hydroxy-B(a)P and a major decrease in the amount of water-soluble metabolites in the extract-treated group. After β-glucuronidase treatment of the aqueous phase, the amount of 9-hydroxy- and 3-hydroxy-B(a)P in the red clover extract-treated group was reduced by 30% and 22%, respectively, when compared to DMSO controls. The water-soluble metabolites were also decreased by 18% in the red clover extract-treated cells. Thus the major effect of red clover extract was to inhibit the formation of B(a)P-phenol glucuronides.

The effect of the crude 95% ethyl alcohol extract on the binding of B(a)P to DNA was also examined (Table 2). At a dose of 250 μg/ml the extract inhibited B(a)P-DNA binding by 30% to 41% compared to controls in three separate experiments. Analysis of the B(a)P-DNA adducts present in enzyme-digested DNA samples by HPLC demonstrated that the extract inhibited the formation of both the syn- and anti-isomers of B(a)PDE. The syn-B(a)PDE-dGuo adduct decreased from 37% to 64% compared to controls, and the (+)-anti-B(a)PDE-dGuo adduct decreased from 48% to 75%.
INHIBITION OF B(a)P METABOLISM BY BIOCHANIN A

**Trifolium pratense (Leguminosae)**

918.0 g fresh plant

Blended with 95% EtOH

Filtered and concentrated in vacuo

*95% EtOH fraction 46.5 grams

Partition 37.4 g between CHCl₃, H₂O, MeOH (10:9:1)

Concentrate in vacuo

*CHCl₃ fraction, 12.6 g

Interface, 1.56 g

H₂O fraction, 23.4 g

Chromatograph 1.96 g on silica gel flash column

Biochanin A, 0.47 g

Fraction #: 1A 1B 1C 1D 1E 1F 1G 1H 1I

Weight, mg: 18.6 224.3 26.1 341.6 23.8 27.5 283.4 657.6 202.9

Chromatograph 245 mg on Silica Gel Rotor, Chromatotron

Fraction #: 2A 2B 2C 2D 2E 2F 2G

Weight, mg: 12.5 110.8 16.0 59.0 11.6 34.0 10.5

Chromatograph 54.0 mg on Silica Gel Rotor, Chromatotron

Fraction #: 3A 3B 3C

Weight, mg: 2.7 40.8 6.5

Biochanin A, 30 mg

* Active (greater than 20% difference) at dose-response dose.

# Active at two times (2x) dose-response dose.

The effect of biochanin A on the binding of B(a)P to DNA in hamster embryo cell cultures was also examined. Biochanin A caused a 54% decrease in B(a)P metabolism at 25 μg/ml. After exposure of cultures to 25 μg of biochanin A and 1 μg of [³H]B(a)P per ml of medium for 24 h, biochanin A treatment reduced the amount of B(a)P bound per mg of DNA from 74.3 pmol in the control group to 35.1 pmol in the biochanin A group in one experiment and from 72.2 to 45.4 in a second experiment. Thus, biochanin A inhibited the binding of B(a)P to DNA to an extent similar to that obtained in the crude extract (Table 2).

**DISCUSSION AND CONCLUSIONS**

There are several bioassays which are under investigation for the detection of compounds suspected of having potential cancer chemopreventive activity. Antimutagenic activity, in the form of an anti-Ames assay, has been commonly used in the United States and Japan (13, 14). Mitscher et al. (15) used this bioassay to isolate and identify glabrene, a known isoflavene exhibiting antimutagenic activity. Nishino et al. (16) used the antitumor-promoting activity of glycyrrettic acid against 7,12-dimethylbenz(a)anthracene and teleocidin as a model of cancer prevention. The decrease in formation of carcinogenic N-nitroso compounds produced by α-tocopherol and ascorbic acid was used as a criterion for chemoprevention by Narkus et al. (17) and Mervish (18). Sakiyama et al. (19) used the inhibition of transformation of the mouse 10T½ cell line induced by X-ray or N-methyl-N'-nitro-N-nitrosoguanidine as a model to show the anticarcinogenic effects of lipopolysaccharides and indomethacin. The induction of aryl hydrocarbon hydroxylase activity in liver and intestinal mucosa of Sprague-Dawley rats was used by Wattenberg et al. (3) to isolate and identify a group of indoles from cruciferous vegetables (20, 21). Another screen by Wattenberg et al. (5) used the induction of glutathione S-transferase activity, a major detoxification enzyme system, for a number of electrophiles, including many carcinogens, in mouse liver and intestinal mucosa to isolate a group of known diterpenes from green coffee beans.

The screening procedure described in this paper measures effects on the ability of hamster embryo cell cultures to metabolize the carcinogen B(a)P. Induction of inhibition of B(a)P metabolism of treated cultures by >20% as compared with control cultures was considered to be an active test. The altered pattern of metabolism was determined by HPLC analysis of the B(a)P metabolites formed, and the effects on binding of B(a)P to DNA are determined. Confirmed active extracts are then fractionated using the bioassay as a guide. Advantages of our method are that activity data can be generated within a few days after the extract or compound is tested, and a large number of test compounds can be screened in a short period of time.

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INHIBITION OF B(a)P METABOLISM BY BIOCHANIN A

Table 1 Activity of fractions in B(a)P metabolism assay

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Dose-response dose (μg/ml medium)</th>
<th>% of change from control</th>
<th>2× dose-response dose</th>
<th>% of change from control</th>
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<tr>
<td>95% Ethyl alcohol</td>
<td>500</td>
<td>-39.9 ± 6.2 b</td>
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<td>-68 ± 4.4 e</td>
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<tr>
<td>CHCl3</td>
<td>750</td>
<td>-40.4 ± 6.9 e</td>
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<td>-33.8 ± 4.6 e</td>
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<tr>
<td>Interface H2O</td>
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<td>-35.6 ± 5.7 e</td>
<td>406</td>
<td>-34.6 ± 4.6 e</td>
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<tr>
<td>CH3</td>
<td>25.2</td>
<td>-17.7 ± 7.3</td>
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<td>-8.0 ± 15.2</td>
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<td>3.9</td>
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<td>17.2 ± 4.6</td>
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<tr>
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<td>-12.9 e</td>
<td>32.2</td>
<td>-32.7 ± 6.4</td>
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<tr>
<td>2A</td>
<td>2.3</td>
<td>-13.8 e</td>
<td>4.6</td>
<td>-12.9 ± 6.4</td>
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<tr>
<td>2B</td>
<td>8.6</td>
<td>-25.3 ± 6.4</td>
<td>17.2</td>
<td>-19.9 ± 6.4</td>
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<tr>
<td>2C</td>
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<td>9.8</td>
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<td>-23.0 e</td>
</tr>
<tr>
<td>2F</td>
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<td>-6.8 re</td>
<td>0.8</td>
<td>-23.0 e</td>
</tr>
<tr>
<td>3A</td>
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<td>-23.7 ± 6.4</td>
<td>12.8</td>
<td>-23.0 ± 6.4</td>
</tr>
<tr>
<td>3B</td>
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<tr>
<td>Biochanin A</td>
<td>4.7</td>
<td>-12.2 e</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>9.5</td>
<td>-32.1 e</td>
<td></td>
<td></td>
</tr>
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<td></td>
<td>19.0</td>
<td>-47.9 e</td>
<td></td>
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<tr>
<td></td>
<td>23.6</td>
<td>-48.8 e</td>
<td></td>
<td></td>
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* Mean ± SD of 3 experiments.
b Active (greater than 20% difference) at dose-response dose.
* Average ± range of 2 experiments.
* One experiment.
* Active at 2× dose-response dose.
* Mean ± SD of 4 experiments.

Fig. 2. The amount of B(a)P metabolites formed in hamster embryo cell cultures in the presence or absence of 500 μg/ml of red clover crude extract. The cultures were treated, and the medium samples analyzed as described in "Materials and Methods." Columns, mean for 3 flasks per group; bars, SD. *, red clover extract-treated samples that differed significantly from the corresponding control (based upon Student's t test; P < 0.01). +, control; L. pratense (b-Glucuronidase); L. pratense (b-Glucuronidase).

Fig. 3. Isoflavones isolated from red clover leaves and flowers (25, 26).

of different samples can be screened simultaneously. After pure active compounds are isolated and their effect on the metabolic activation of B(a)P is established, they will then be further tested using in vivo bioassays to determine their effect on tumor induction by various classes of carcinogens. These in vivo bioassays are essential for determining whether a compound acts as an anticarcinogen and against which classes of carcinogens it was active.

Thus far we have screened over 70 species and varieties of plants and vegetables comprising 27 families. One of the first plant extracts demonstrated to produce reproducible inhibition of B(a)P metabolism in the hamster cell culture assay was that prepared from red clover. Based upon inhibition of B(a)P metabolism the crude red clover extract was fractionated, and
and isolate pure compounds with potential anticarcinogenic activity. Measuring modifiers of carcinogen metabolism that influence genotoxic activity is important for understanding carcinogenesis. Various types of short-term assays may be anticipated to detect genotoxic effects. A study with B(a)P, a known carcinogen, demonstrated that genistein, an isoflavone related to biochanin A, showed genotoxic activity (3). 7,8-Benzoflavone, a synthetic flavonoid, is an inhibitor of microsomal mixed-function oxidases and inhibits tumor induction by hydrocarbons in animals.

The authors would like to thank David Burns and Nancy Hall for their contributions to this study.

### Table 2: B(a)P-DNA binding in hamster embryo cells treated with extracts of red clover

<table>
<thead>
<tr>
<th>Experiment</th>
<th>% of water-soluble B(a)P DNA adducts (pmol/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.4 4.9</td>
</tr>
<tr>
<td>Test</td>
<td>12.7 4.9</td>
</tr>
<tr>
<td>Binding of B(a)P to DNA:</td>
<td>8.4 3.0</td>
</tr>
</tbody>
</table>

A pure active compound, biochanin A, was isolated which produced an inhibition of B(a)P metabolism of 30 to 50% at 9.5 to 23.6 μg/ml compared to DMSO controls. Exposure of hamster embryo cell cultures to biochanin A at a dose of 25 μg/ml of medium resulted in a 37 to 50% inhibition in the binding of B(a)P to DNA. This compound appears to be one of the major components responsible for the inhibition of B(a)P-DNA interactions by the red clover extract. The strong correlation between the binding of aromatic hydrocarbons to DNA and their carcinogenic activity suggests that biochanin A is a good candidate for further testing to measure inhibition of tumor induction by hydrocarbons in animals.

Several flavonoids have been shown to possess anticarcinogenic activity (3). 7,8-Benzoflavone, a synthetic flavonoid, is an inhibitor of microsomal mixed-function oxidases and inhibits the metabolism, binding to DNA, and tumorigencsis of 7,12-dimethylbenz[a]anthracene in mouse skin (22). This same flavonoid also inhibits the metabolism of B(a)P in rat hepatic microsomes that have been induced with 3- methylcholanthrene (23). Huang et al. (24) examined 28 flavonoids for their effect on mutagenicity of anti-B(a)PDE in *Salmonella* and found that 8 had significant anticarcinogenic activity. Interestingly, one of the flavonoids found to be inactive (50% inhibitory dose > 100) was genistin, an isoflavone related to biochanin A (see Fig. 3) and a minor constituent of red clover (25, 26). Since the compound tested [B(a)PDE] was an ultimate mutagenic metabolites of B(a)P, that assay would not be expected to detect compounds that alter metabolic activation of B(a)P. Thus, various types of short-term assays may be anticipated to detect anticarcinogens that work by different mechanisms. In view of the requirement of the majority of classes of chemical carcinogens for metabolic activation and the ability of the metabolism assay to measure changes in enzymes both involved in activation as well as detoxification, the hamster cell assay should be capable of detecting modifiers of carcinogen metabolism that act by a number of mechanisms. The results demonstrate that the effects of test compounds on B(a)P metabolism and DNA binding in hamster embryo cell cultures can be used to screen and isolate pure compounds with potential anticarcinogenic activity from plants and other natural products.

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### References


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