

# Confirmation of Uterotrophic Activity of 3-(4-Methylbenzylidene)camphor in the Immature Rat

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In this study we found that the ultraviolet sunscreen component 3-(4-methylbenzylidene)camphor (4MBC) is uterotrophic in immature rats when administered by either subcutaneous injection or oral gavage. These data confirm earlier reports of uterotrophic activity for this agent when administered to immature rats in the diet or by whole-body immersion; however, they are in contrast to negative unpublished immature rat uterotrophic assay results. Data also indicate that 4MBC binds to isolated rat uterine estrogen receptors and shows activity in a human estrogen receptor yeast transactivation assay; however, we considered both of these effects equivocal. In this study, we confirmed the original observation that 4MBC was active as a mitogen to MCF-7 breast cancer cells. We evaluated and discounted the possibility that the estrogenic activity of 4MBC is related to its bulky camphor group, which is of similar molecular dimensions to that of the weak estrogen kepone. Uncertainty remains regarding the mechanism of the uterotrophic activity of 4MBC. **Key words:** estrogenic activity, ER binding, immature rat uterotrophic assay, MCF-7 cell proliferation, 3-(4-methylbenzylidene)camphor, yeast ER transactivation. *Environ Health Perspect* 110:533–536 (2002). [Online 4 April 2002]

<http://ehpnet1.niehs.nih.gov/docs/2002/110p533-536tinwell/abstract.html>

Schlumpf et al. (1,2) have reported the ultraviolet sunscreen component 3-(4-methylbenzylidene)camphor (4MBC; structure shown in Figure 1) to be uterotrophic when administered either in diet to immature Long-Evans rats or by whole-body immersion of hairless *hrl/hr* immature rats in an oil solution. Subsequently, Bolt et al. (3) questioned the validity of those data and referred to two negative unpublished immature rat uterotrophic assays of 4MBC.

Our interest in 4MBC is to discern the active uterotrophic species involved; 4MBC is devoid of a hydroxyl group but possesses the large camphor group, both of which argue *a priori* against estrogenicity. When the discussion between Bolt et al. (3) and Schlumpf et al. (4) appeared, we had already studied the binding properties of 4MBC to isolated rat uterine estrogen receptors (ER), its activity in a yeast ER-transactivation assay, and its activity in both oral and subcutaneous immature rat uterotrophic assays. Our data appear to be relevant to that discussion (3,4), which we describe here together with a repeat of the MCF-7 breast cancer cell proliferation assay of 4MBC described by Schlumpf et al. (4).

## Materials and Methods

**Chemicals.** We obtained 4MBC (CAS no. 38102-62-4; Merck batch no. TT805785 029) from ChemQuest (Wilmslow, Cheshire, UK), and 17 $\beta$ -estradiol ( $E_2$ ), HEPES buffer, arachis oil (AO), diethylstilbestrol (DES), and (1*R*)-(+)-camphor (99%; structure shown in Figure 1) from Sigma Chemical Company (Poole, Dorset, UK). ChemService (West

Chester, PA, USA) supplied kepone (98%; structure shown in Figure 1), and Amersham Life Sciences (Bucks, UK) supplied (2,4,5,7- $^3H$ )-estradiol ( $^3H$ - $E_2$ ; 88 Ci/mmol). We obtained dimethyl sulfoxide (DMSO) from Fisher Scientific (Loughborough, Leicestershire, UK) and chlorophenol red- $\beta$ -galactopyranoside (sodium salt; CPRG) from Roche Diagnostics (East Sussex, UK).

**ER binding assay.** We conducted the ER competitive binding assay using the cytosol isolated from the uteri of 21–25-day-old female Alpk:APfSD rats (supplied by the animal breeding unit at AstraZeneca Pharmaceuticals, Macclesfield, Cheshire, UK) as described previously (5), using methods based on those of Shelby et al. (6). We incubated uterine cytosol with duplicate dose levels of 4MBC (in 10  $\mu$ L DMSO) and  $^3H$ - $E_2$  (0.22  $\mu$ Ci;  $\approx 5 \times 10^{-9}$  M  $E_2$ ) at 4°C for 17 hr. We then precipitated the receptor ligand with hydroxylapatite, washed it well, and suspended it in Optiphase (LKB Scintillation Products; FSA Laboratory Supplies, Leics, UK) for the determination of radioactivity in a Packard Tri-carb 2100 TR liquid scintillation analyzer (Packard Instruments, Pangbourne, UK). We tested 10-fold dilutions of  $E_2$  and DES ( $5 \times 10^{-10}$  to  $5 \times 10^{-6}$  M) and 4MBC ( $5 \times 10^{-10}$  to  $5 \times 10^{-4}$  M). We conducted two experiments, each with duplicate dose levels of compounds and of vehicle alone (DMSO) to ascertain a 100% binding value. We determined the top dose level of 4MBC tested ( $5 \times 10^{-4}$  M) by the apparent limit of its solubility in the assay medium.

**Yeast ER transactivation assays.** We obtained the recombinant *Saccharomyces*

*cerevisiae* yeast strain harboring the human ER from J. Sumpter (Brunel University, Uxbridge, Middlesex, UK). The DNA sequence encoding the human ER was integrated into the genome of the *Saccharomyces cerevisiae* yeast strain that also contained an expression vector in which an ER response element was cloned upstream of the reporter gene *LacZ* (encoding the enzyme  $\beta$ -galactosidase) (7). All glassware used in these assays was dedicated purely for this purpose. We prepared media components (8) and performed the yeast assays as described previously by Routledge and Sumpter (7). We serially diluted test chemicals and negative controls in ethanol (except for the vehicle control, HEPES, which we diluted in aqueous media). The top doses used in the assay were  $10^{-3}$  M for camphor, 4MBC, and HEPES (control);  $10^{-5}$  M for kepone; and  $10^{-8}$  M for  $E_2$ .

We transferred 10  $\mu$ L aliquots to 96-well flat-bottom microtiter plates (Linbro/Titertek, ICN FLOW, Bucks, UK) and allowed to evaporate to dryness. We then dispensed 200  $\mu$ L aliquots of assay medium (i.e., medium containing recombinant yeast and CPRG, the chromogenic substrate) to each sample well. We sealed the plates with autoclave tape and shook them vigorously for 2 min prior to incubation at 32°C for 3 days. At this point, we measured color development of the medium at an absorbance of 540 nm (using a Labsystems iEMS Reader MF plate reader; Labsystems Affinity Sensors, Cambridge, UK). In addition, we measured absorbance at 620 nm to assess turbidity (growth/viability of the yeast). If the turbidity measurement was decreased > 10% in any sample, we discarded this result because of deleterious effects of the test chemical on yeast growth and viability.

**Cell proliferation assay.** MCF-7 cells (a gift from M. Parker, Imperial Cancer Research Fund, London) in RPMI 1640

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media (containing phenol red) supplemented with 10% (v/v) fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. For the cell proliferation experiments, we trypsinized MCF-7 cells and suspended them in RPMI media (phenol red-free) supplemented with 5% (v/v) charcoal-stripped fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. We then seeded them into 24-well plates at an initial concentration of 30,000 cells/well. We cultured MCF-7 cells under these conditions for 5 days, then incubated them with the appropriate concentration of test compound for 6 days (media changed every 3 days), as described by Schlumpf et al. (4). We harvested cells by trypsinization on day 6 and counted them visually using a hemocytometer. We evaluated statistical significance relative to vehicle (ethanol) control by a two-sided Student's *t*-test ( $p < 0.05$  or  $p < 0.005$ ).

**Animals and dosing.** We obtained 19- to 20-day-old (38–50 g) female Alpk:APfSD (Wistar-derived) rats from the barriered animal breeding unit (AstraZeneca). The animals were housed six per cage in solid-bottom cages and allowed to acclimatize for 24 hr. They were fed Rat and Mouse No. 1 (RM1 diet; Special Diet Services Ltd., Witham, Essex, UK) and water *ad libitum* for the duration of the study. We homogenized 4MBC in AO for at least 5 min using an Ultra Turrax homogenizer (Janke and Kunkel, IKA-Labortechnik, Germany). We stored dosing solutions at room temperature and shook them vigorously prior to dosing. We used a dosing volume of 5 mL/kg body weight for both routes of chemical administration.

We conducted preliminary dose setting studies on 4MBC by exposing weanling rats to 4MBC for 3 days, as in the proposed uterotrophic assays. We defined the maximum tolerated dose (MTD) for the oral gavage studies as 1,000 mg/kg based on the absence of an increase in body weight on the third day of dosing. We therefore used dose levels equivalent to 80% (800 mg/kg) and 50% (500 mg/kg) of the MTD for the oral gavage studies. We observed no toxicity for 4MBC up to 1,000 mg/kg by subcutaneous injection, so we used dose levels of 500 mg/kg and 1,000 mg/kg for the subcutaneous injection study. Each female received a single oral or subcutaneous injection of the appropriate compound at the appropriate dose level on each of 3 days (postnatal days 20–21 to 23–24). We used DES as a positive control agent for the uterotrophic assays (5 µg/kg dissolved in AO).

During the uterotrophic assays we modified the test protocol so that animals exposed to 800 mg/kg 4MBC by oral gavage received moistened diet from day 3 of study. We made this change in protocol because these animals were failing to eat the pelleted diet

on the third day of dosing and thereby showed signs of toxicity. Moistening the diet led to a resumption of normal feeding.

**Uterotrophic assays.** All animals were terminated by an overdose of fluothane (AstraZeneca Pharmaceuticals) followed by cervical dislocation (9) 24 hr after the last of three daily administrations of the test agents. We removed the uterus, trimmed it free of fat, and gently blotted and weighed it. We then placed the uterus in a preweighed vial, dried it overnight (24 hr) at 70°C, and reweighed it (9). We analyzed data by variance and covariance using the MIXED procedure of SAS (10). Specifically, we analyzed uterine weights by covariance with the terminal body weights. We adjusted terminal body weights for covariance with initial body weights, and assessed differences from control values statistically using a two-sided Student's *t*-test based on the error mean square from the analysis of covariance. We considered the individual to be the statistical unit.

## Results

**ER binding assays.** The data from the ER competitive binding assays are shown in Figure 2. The data points are percentages of the vehicle-alone value and represent the means from the two separate experiments, both of which gave both qualitatively and quantitatively similar results. Both E<sub>2</sub> and DES treatments resulted in the binding characteristics previously reported from this laboratory (5). 4MBC reproducibly displaced E<sub>2</sub> from ER, but no more than 20% displacement could be achieved. We therefore could not determine the concentration at which 50% inhibition occurs. Such instances have been encountered before with phthalate esters (11,12) where, as in this case, the compounds were approaching their aqueous solubility limits; in those cases the authors questioned whether the apparent competitive binding of the substrates to ER was artifactual. The present data therefore provide only equivocal evidence that 4MBC binds competitively to ER.

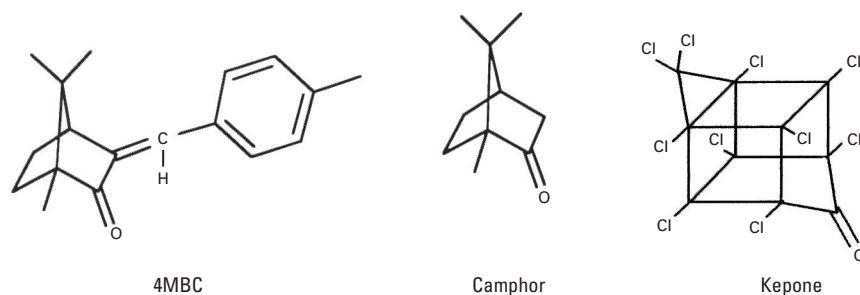


Figure 1. Chemical structures of chemicals used in this study.

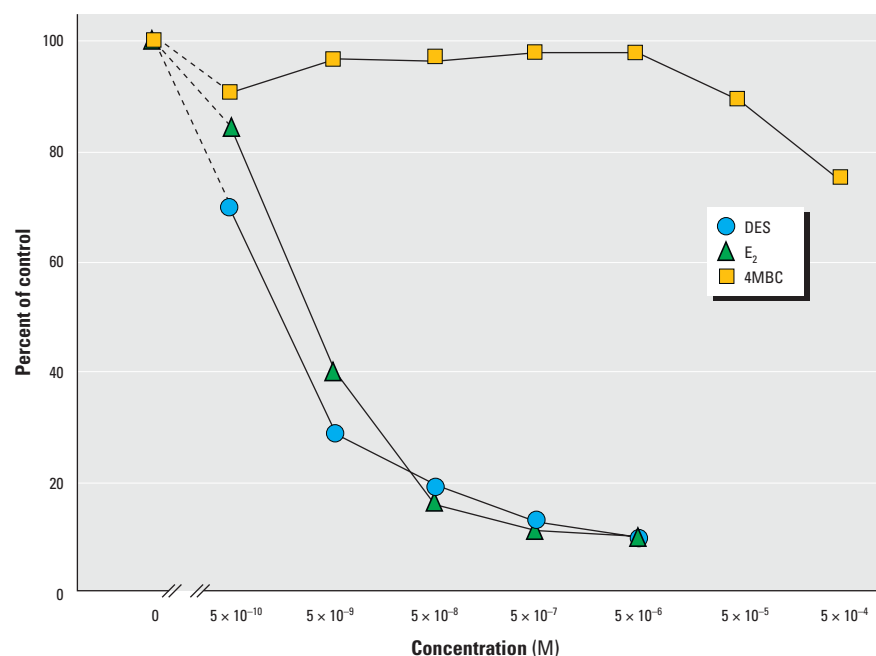


Figure 2. Estrogen competitive binding assay with <sup>3</sup>H-E<sub>2</sub>, DES, and 4MBC using immature rat uterine receptor preparations. The data are percentages of the vehicle (DMSO) control value and are the average of two separate experiments, each of which gave similar results.

**Yeast ER transactivation assays.** The yeast assay data are shown in Figure 3. E<sub>2</sub> gave the expected positive response, and the HEPES vehicle controls behaved as expected (5,7). Camphor was inactive, and kepone showed weak activity in the assay. The toxicity (reduced turbidity) of kepone precluded evaluations of higher doses. 4MBC reproducibly, but only marginally, increased optical absorbance, and we classified this response as equivocal. We found no decreases in turbidity in the 4MBC experiments, suggesting that toxicity had not been a limiting factor. The plateau nature of the assay response to 4MBC suggests that re-solution of the agent into the medium may have become the limiting factor in the assay response.

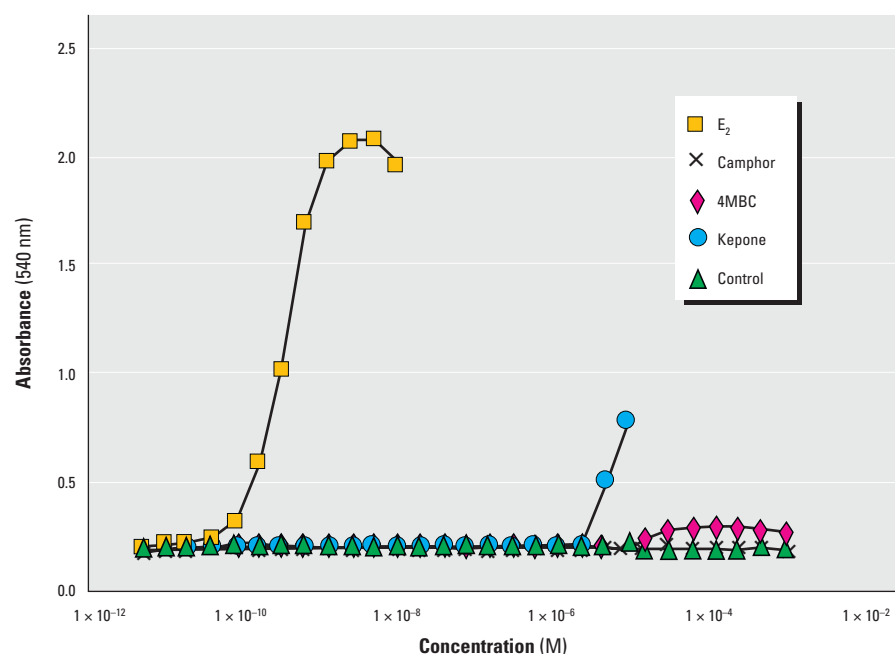
**Cell proliferation assay.** Proliferation of MCF-7 cells was increased by incubation with 1–10  $\mu$ M 4MBC for 6 days in culture (Figure 4). Cell numbers are expressed as mean  $\pm$  SEM for three counts per treatment group on day 6 of treatment. The results are representative of several experiments. At concentrations above  $2 \times 10^{-5}$  M 4MBC, we observed a significant decrease in the number of viable cells (results not shown), indicating that 4MBC may be cytotoxic to these cells at high concentrations. We observed maximal effects on MCF-7 cell proliferation at 10  $\mu$ M 4MBC in this system; these levels of proliferation are similar to those reported for 4MBC by Schlumpf et al. (4). E<sub>2</sub>, at a concentration of  $1 \times 10^{-10}$  M, caused MCF-7 cells to proliferate significantly,

compared to vehicle control ( $1 \times 10^{-10}$  M E<sub>2</sub>;  $1,037,600 \pm 268,493$  cells/well compared to control  $234,920 \pm 36,249$  cells/well;  $p < 0.05$ ).

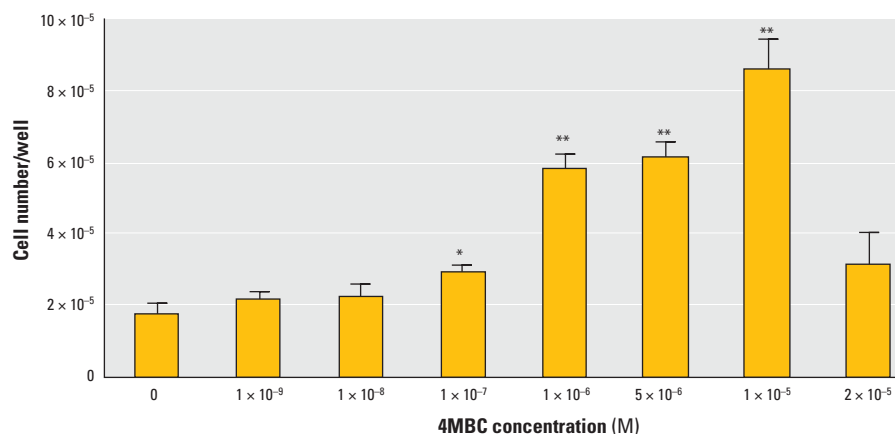
**Uterotrophic assays.** 4MBC was clearly active in the immature rat uterotrophic assay by both routes of exposure (Table 1, Figure 5). Both blotted and dry uterine weights were increased in all test groups ( $p < 0.01$ ). We observed clinical signs of toxicity following the third administration of 800 mg/kg 4MBC by oral gavage. One female from this group was removed from the study shortly after the third and final dose (data not included in final analyses; uterine weight was 63.9 mg). We then placed all females in this group on moistened diet for the remainder of the study. This change of protocol restored normal eating patterns, suggesting that the toxicity observed was caused by, or was exacerbated by, reduced food intake for this group. At termination, we observed a statistically significant reduction in body weight gain in both groups exposed to 4MBC by oral gavage (Table 1). Given the signs of toxicity seen for the highest oral dose level of 4MBC in the main study (but not in the dose-setting study), it would be preferable to rely on the data generated at 500 mg/kg 4MBC (9% reduction in body weight and no clinical signs of toxicity). We saw no indications of toxicity in females exposed to this compound by subcutaneous injection, nor did we see any adverse effects on body weight (Table 1). DES gave the expected (5) increase in uterine weight in the absence of evident toxicity in each experiment (Table 1).

## Discussion

The present results demonstrate that 4MBC is uterotrophic to immature rats when administered by either oral gavage or subcutaneous injection. These data therefore support positive uterotrophic results reported earlier for 4MBC when administered to immature rats, either in diet or by whole-body immersion in an oil solution (1). Within this context, the two sets of unpublished and negative immature rat uterotrophic assay data for 4MBC referred to by Bolt et al. (3) are of interest, but they cannot be discussed further until formally published. The relatively high dose levels of 4MBC required to produce a partial uterotrophic response suggest that it is a comparatively weak rodent estrogen. The mild toxicity seen for the highest oral dose of 4MBC is unlikely to have contributed to the generation of the uterotrophic response, because we saw similar uterotrophic activity at the lower oral dose level. In addition, we observed positive uterotrophic effects in the subcutaneous injection studies, which were in the absence of clinical signs of toxicity or changes in body weight. The present uterotrophic assay data are insufficient to



**Figure 3.** Human ER recombinant yeast assay for E<sub>2</sub>, 4MBC, kepone, and camphor (structures shown in Figure 1). The control solution is HEPES buffer.



**Figure 4.** Effect of 4MBC on the proliferation of MCF-7 human breast cancer cells. Data are shown as the mean  $\pm$  SEM.

\* $p < 0.05$ ; \*\* $p < 0.005$ .

contribute to assessment of the no-effect level of 4MBC to female rats.

Schlumpf et al. (1) were prompted to evaluate 4MBC in the uterotrophic assay based on its clear activity in the MCF-7 cell proliferation assay in the dose range of  $5 \times 10^{-6}$  to  $5 \times 10^{-5}$  M. The authors also demonstrated both the induction of the estrogen responsive pS2 protein by 4MBC in MCF-7 cells and the blockage of 4MBC-induced MCF-7 cell proliferation by the antiestrogen ICI 182,780. In the present ER binding and yeast ER transactivation assays, we observed only equivocal evidence for estrogenic activity for 4MBC, and then only in the higher dose ranges of  $5 \times 10^{-5}$  to  $5 \times 10^{-4}$  M (ER binding) and  $3 \times 10^{-5}$  to  $10^{-3}$  M (yeast assay). In the last two dose ranges, insolubility may have precluded an accurate assessment of the activity of 4MBC. In contrast, we confirmed the clear activity reported for 4MBC at  $5 \times 10^{-6}$  M in the MCF-7 assay (4) in the present experiments. This difference in assay outcomes between the MCF-7 assay and the yeast assay of 4MBC may be related to technical differences in the respective assay protocols: in the MCF-7 assay, we added the test agents in medium to

the microwells, whereas the yeast assay protocol allows an ethanol solution of the test agent to evaporate to dryness in the microwells, followed by addition of the yeast cells in medium (7,8,12). Alternatively, metabolic transformation of 4MBC to an estrogenic species may be occurring in the MCF-7 cells but not in the yeast cells.

We also considered whether the estrogenic activity of 4MBC may be associated with its bulky terminal camphor group, this being similar in shape to the weak uterotrophic agent kepone (Figure 1) (6). On the basis of "Dreiding" stereo-models, kepone and camphor show similarities both in their molecular non-space-filling dimensions and in their three-dimensional shape (data not shown). However, although kepone was active in the yeast transactivation assay, camphor was inactive. These observations weaken a possible structural relationship of 4MBC to kepone.

In summary, 4MBC shows activity as a weak uterotrophic agent to the immature rat and mitogenic activity to MCF-7 cells treated *in vitro*. However, we observed only equivocal evidence of estrogenicity *in vitro* in ER binding and yeast transactivation assays. This

unusual combination of assay responses indicates two main possibilities: first, that 4MBC is metabolized to an estrogenic intermediate in MCF-7 cells and in rats but not in yeast cells or in isolated uterine cytosol; second, that the mechanism by which 4MBC elicits its activity in MCF-7 cells and in rats is independent of the ER. In connection with the latter possibility, it is interesting to note that in subchronic studies of 4MBC, high doses were shown to interfere with thyroid hormone regulation, causing triiodothyronine ( $T_3$ ) levels to increase despite maintenance of normal thyroxine ( $T_4$ ) levels (13). Therefore, changes in these hormones may be induced by 4MBC, which may be responsible for its apparently selective estrogenic activities. As suggested by Schlumpf et al. (1,2,4), analysis of 4MBC in rodent reproductive and developmental assays will be necessary to advance assessment of its potential endocrine toxicity to humans.

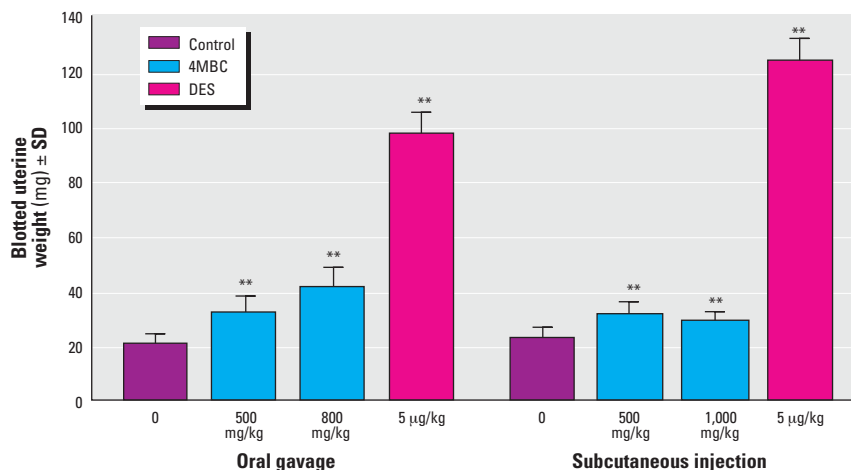
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**Table 1.** Activity of 4MBC in the immature rat uterotrophic assay.

| Compound           | Dose (kg) | No. animals     | Uterine weight ±SD (mg) |              | Terminal body weight ± SD (g) |
|--------------------|-----------|-----------------|-------------------------|--------------|-------------------------------|
|                    |           |                 | Blotted                 | Dry          |                               |
| Oral gavage route  |           |                 |                         |              |                               |
| AO                 | 5 mL      | 12              | 22.0 ± 2.5              | 4.5 ± 0.4    | 55.9 ± 3.9                    |
| 4MBC               | 500 mg    | 12              | 32.5 ± 6.5**            | 6.8 ± 1.0**  | 51.3 ± 4.8*                   |
|                    | 800 mg    | 11 <sup>a</sup> | 42.4 ± 6.0**            | 9.3 ± 3.0**  | 47.6 ± 4.4**                  |
| DES                | 5 µg      | 5               | 97.3 ± 7.7**            | 17.0 ± 1.4** | 56.8 ± 4.1                    |
| Subcutaneous route |           |                 |                         |              |                               |
| AO                 | 5 mL      | 12              | 23.3 ± 4.3              | 4.9 ± 0.8    | 56.4 ± 3.2                    |
| 4MBC               | 500 mg    | 12              | 31.1 ± 4.6**            | 6.0 ± 0.7**  | 56.9 ± 5.2                    |
|                    | 1,000 mg  | 12              | 29.4 ± 3.5**            | 5.6 ± 0.7**  | 56.5 ± 4.1                    |
| DES                | 5 µg      | 5               | 123.6 ± 8.2**           | 20.9 ± 1.7** | 56.4 ± 5.3                    |

<sup>a</sup>We removed one female from this group 2 hr after the final dose because of clinical signs of toxicity. The uterus was removed at that time and weighed as 63.9 mg (blotted uterine weight). \* $p < 0.05$ ; \*\* $p < 0.01$ .



**Figure 5.** 4MBC induces a significant increase in blotted uterine weight when given to rats by either oral gavage or subcutaneous injection; 500 mg/kg and 800 mg/kg are equivalent to 50% and 80% of the oral MTD. We did not observe toxicity at doses up to 1,000 mg/kg when exposure was by subcutaneous injection. The positive control agent, DES, gave the expected uterotrophic response. AO was used as the negative control. \*\* $p < 0.01$ .