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Lack of Effect of Trichloropropene Oxide on Benzo(a)pyrene Tumor-Initiating Activity on Mouse Skin*

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Abstract—*The potent epoxide hydratase (EH) inhibitor, 1,1,1,-trichloro-2,3-propene oxide (TCPO), did not enhance the tumor-initiating ability of benzo(a)pyrene (BP) when applied simultaneously with BP in a two-stage system of tumorigenesis in male Swiss mice. BP* (0.1 μmole) *with or without TCPO* (2 μmole) *was applied either once* (day 1) *or twice* (days 1 *and* 3) *to the skin of the backs of mice shaved* 24 hr *before; thereafter the mice were given weekly applications of croton oil* (50 μl 1% *in acetone per mouse for* 50 weeks). *Skin tumors started to appear* 15 weeks *after initiation of the mice, and at the same time in all groups. The experiment was terminated* 50 weeks *after application of carcinogen.*

The skin microsomal epoxide hydratase (48–52 pmole *BP*-4,5 diol/min/mg) *was inhibited* 3 hr *after application of BP alone or BP plus TCPO. No inhibition was observed in microsomes isolated* 16 *or* 24 hr *after application of carcinogen or carcinogen plus epoxide hydratase inhibitor. TCPO has no* in vitro *effect on the* ^{3}H-*BP binding to epidermal DNA* 24 hr *after application of benzo(a)pyrene. Rapid trapping of TCPO by intracellular glutathione and/or nucleophiles would explain this absence of effect, which contradicts the results of D. L. Berry and other* (J. Nat. Cancer Inst. *Vol.* 58, *No.* 4, 1051–1055, 1977).

INTRODUCTION

THE TWO-STAGE system (initiation and promotion) on skin carcinogenesis in mice has been demonstrated [1]. The initiation phase requires only a simple application and is irreversible while the promotion phase requires multiple applications.

Benzo(a)pyrene (BP) is a potent initiator of skin tumors in a two-stage system of carcinogenesis in the mouse [1]. When applied to mouse skin this carcinogen is metabolized by mixed-function oxidases (MFO), in particular by aryl hydrocarbon hydroxylase (AHH), which is localized in the microsomes and nuclear envelope [2–7].

The enzymatic oxidation of benzo(a)pyrene is believed to occur via an initial epoxidation reaction. The highly reactive arene oxides thus formed either interact with macromolecular tissue constituents or are metabolized or rearranged to dihydrodiols, phenols, diol-epoxides or conjugated with glutathione [8–10]. The epoxides seem to be the active intermediates for benzo(a)pyrene. One of the principal metabolites that have been found to react with the DNA of mouse skin epidermis treated with BP is *trans*-7,8-dihydro-7,8-dihydroxy-B(a)P-9, 10-oxide (BP-7,8-diol-9,10-epoxide) [11, 12].

The mixed-function oxidase enzymes are responsible for both the formation and detoxification of the active oncogenic intermediates [3, 8]. The modifiers of MFO may exert certain effects on the enzymes that activate and detoxify polycyclic aromatic hydrocarbons (PAH) [13, 14] and other effects on polycyclic hydrocarbon carcinogenesis [15]. The topical application of 7,8-benzoflavone, a known inhibitor of AHH, did not inhibit the binding *in vivo* of BP to epidermal DNA and tumor initiation [15]. The potent inhibitor of epoxide hydratase (EH), 1,1,1,-trichloro-propene oxide (TCPO), was found to be effective in increasing the skin tumor

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The abbreviations used in this report are: BP, benzo(a)pyrene; AHH, aryl hydrocarbon hydroxylase; EH, epoxide hydratase; MC, 3-methylcholanthrene; TCPO, 1,1,1-trichloropropene-2,3-oxide; 7,8,-Diol, ±(trans)α7β7 dihydroxy-7,8 dihydro-BP; Diol-Epoxide, ±7α,8β-dihydroxy-9β, 10β epoxy-7,8,9,10 tetrahydro-BP.

initiating ability of BP [16] and MC [17]. Others found that in the presence of liver microsomes *in vitro*, TCPO increased BP binding to exogenous DNA [14] and completely inhibited the formation of BP diols [13, 14]. It was also reported that in an epidermal homogenate the BP binding to DNA increased slightly in the presence of TCPO [13]. High-pressure liquid chromatography (HPLC) revealed that BP metabolism by epidermal homogenates resulted in essentially no dihydrodiols [18].

We report here that the potent epoxide hydratase inhibitor TCPO: (a) has no effect *in vivo* on BP–DNA formation, (b) had no effect on skin tumor initiation by benzo(a)pyrene and (c) increased *in vitro* covalent binding of BP to exogenous DNA.

MATERIALS AND METHODS

Chemicals

1,1,1-Trichloropropene 2,3-oxide (TCPO) was product of Aldrich Chemical Company, Milwaukee, WI. [^{3}H) BP (specific activity, 25 Ci/nmole) was purchased from the Radiochemical Centre, Amersham, England; purified on 0.25-mm-thick silica gel (MERCK AG, Darmstadt, Germany) with benzene and diluted with nonradioactive purified BP just prior to use. [^{3}H] benzopyrene-4,5-oxide were prepared in this laboratory (P. Dansette). All other chemicals used were as previously noted [4].

Tumorigenesis experiments

Male Swiss mice selected from the strains maintained by the animal section of IRSC (Villejuif, France) and weighing 18–22 g were used. The animals were fed a commercial diet and water *ad libitum* throughout the entire study. They were shaved with electric clippers 1 day before application of the chemicals. Twenty-five mice were used in each group in the tumorigenesis experiments. Benzo-(a)pyrene (100 nmole) in 0.2 ml acetone was applied topically to the backs of the mice. TCPO (2×10^3 nmole) per 0.2 ml was dissolved in the carcinogen solution. The control group received acetone alone. One type of tumorigenesis experiment was performed: the carcinogen with or without the inhibitor was applied one or two times only on days 1 and 3. This treatment was followed by a weekly application of 20 μl of 1% croton oil in acetone for 50 weeks.

The mice were periodically checked for weight gain or loss, and no significant differences between groups were observed throughout the study. Skin tumor formation was recorded every week and tumors greater than 1 mm in diameter were included in the cumulative total only if they persisted for 3 weeks or more. At intervals during the experiment and at the termination of the study, histopathological examinations were made on all tumors and skin lesions and on selected internal organs.

In vitro ^{3}H-*BP binding studies to DNA*

Each incubation mixture in the *in vitro* binding assay contained the following components in a final volume of 2.5 ml: 0.5 M Tris–HCl, pH 7.5; 2×10^{-3}M NADPH; 3 mmole $MgCl_2$; 0.2 ml microsomal suspension (5 mg of protein) 3 mg DNA and 100 nmole of ^{3}H-BP in 50 μl of acetone. TCPO was added before the microsomes at different concentrations (10^{-6}–10^{-3}M). After incubation at 37°C for 20 min in subdued light, the reaction was stopped by cooling the solution. The medium was made 6% sodium *p*-aminosalicylic acid and 1% sodium dodecyl sulfate (SDS), and DNA was extracted and purified as described previously [4].

In vivo ^{3}H-*BP binding studies to DNA*

In the *in vivo* binding studies, the skins of eight mice that were treated with carcinogen, with or without inhibitor, were scrapped as described previously [19] and homogenized in 7 ml of 6% sodium *p*-aminosalicyclic acid in a tight-fitting Potter–Elvehjem homogenizer. Sodium dodecyl sulfate (SDS) was added to a 1% concentration and the medium was deproteinized with Kirby's phenol mixture [4]. Epidermal DNA was isolated, purified and the radioactivity was measured as described previously [4].

Enzyme assay

AHH was measured according to Hayakawa [20] and the epoxide hydratase (EH) according to Jerina [21]. The reaction medium contained 75 μl Tris–HCl (pH 9) with an appropriate quantity of enzyme. The reaction was started by adding 10 nmole (105 counts/min) [^{3}H] BP-4,5-oxide in 3 μl of CH_3 CN containing 1% NH_4OH. After incubation at 37°C for 60 min, the reaction was stopped by the addition of 45 μl of tetrahydrofurane, and the mixture was shaken with a vortex and cooled in ice. Forty microlitres of this incubation medium was spotted on chromatographic sheets Quantum LQDF (20 × 5 cm). The chromatograms were allowed to develop in benzene–methanol (9:1) for a dis-

tance of 15 cm or until the R_f of diol was 0.4. The diol was scraped off and eluted in 1 ml MeOH and counted in 10 ml of a toluene scintillation fluid.

RESULTS

It has been shown that TCPO at high concentrations (2×10^{-3}M) is a potent inhibitor of epoxide hydratase *in vitro* [13, 14, 22] and has a selective effect on the metabolism of BP. TCPO completely prevented the formation of all three diols of BP, while the formation of 3-OH and 9-OH-BP was not affected [13]. It is possible that in addition to its principal inhibitory action on the hydratase activity TCPO might have had a selective effect on the oxidase. Previous studies *in vitro* have indicated an increased BP-binding DNA in the presence of TCPO with liver nuclei and microsomes [4, 14, 17].

However, using epidermal homogenates, Berry *et al.* [16] showed that the epoxide hydratase inhibitor, TCPO, at concetrations from one-fourth to eight times the concentration of the BP only slightly increased the *in vitro* covalent binding of the BP to exogenous DNA.

When TCPO was added in our experiments to the skin microsomes at concentrations higher than 10^{-4} (Fig. 1), the binding of ^{3}H-BP to DNA increased. Since EH inhibitors have enabled the isolation of K-region epoxides from the *in vitro* metabolism experiments of a number of hydrocarbons [8], the increased binding was probably through an epoxide intermediate [13, 14]. This would suggest that, although TCPO may inhibit overall oxygenation of BP, the binding *in vitro* of BP to DNA was largely through an epoxide intermediate whose amount was increased by the inhibition of the epoxide hydratase.

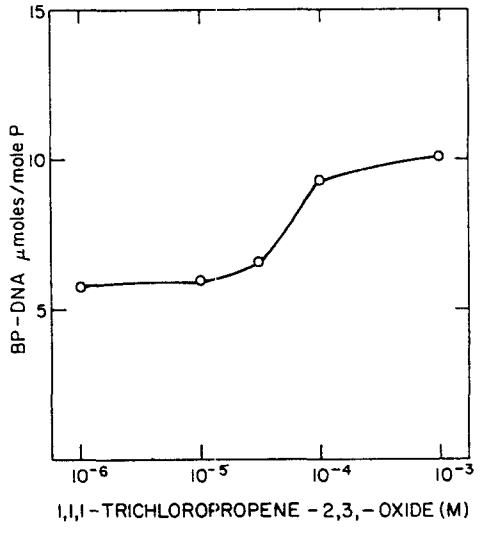

Fig. 1. The effect of TCPO on ^{3}H-BP binding to exogenous DNA in the presence of skin microsomes. ^{3}H-BP was incubated with microsomes (5 mg protein) obtained from the skin of mice in the presence of increasing amounts of TCPO (see Materials and Methods). Each value represents 2 experiments. S.D. *for each point was less than* 10%.

Our results concerning the *in vivo* effect of TCPO on skin microsomal EH are presented in Table 1. They were obtained with a single application of the carcinogen BP (100 nmole per mouse) with or without 2×10^3 nmole of TCPO. Mice were killed 0, 3, 12 and 24 hr after treatment. We chose this time because the AHH is inducible during this period with a maximum at 12 hr after treatment [16]. The results show that *in vivo* TCPO had no inhibitory effect on EH at the times indicated. The apparent inhibition obtained at 3 hr was also observed in the group treated with BP only and could be due to the presence of some BP oxides acting as competing substrates for the hydratase. TCPO also had no effect *in vivo* in the binding of ^{3}H-BP to epidermal DNA at the same conditions (Table 2). These results do not correspond to those obtained after incubation of the *in vitro* system using liver microsomes [14, 17], nuclei [4] or skin microsomes (Fig. 1).

Table 1. Epoxide hydratase in microsomal preparations from mouse skin pretreated with benzo(a)pyrene and TCPO

Treatment (nmole)	Specific activity† 0 hr	3 hr	16 hr	24 hr
Acetone	60	48	52	40
BP (100)*	50	19	54	70
PB (100)+ TCPO (2000)	<10	32	59	47

The mice were treated with benzo(a)pyrene in a manner identical to the procedure used in the tumorigenesis experiment in which the carcinogen was applied only once. The enzyme activity was measured 0, 3, 16 and 24 hr after application of BP and TCPO.

*All compounds were applied on 0.2 ml acetone.

†pmoles of BP-4.5-dihydrodiol formed per mg of protein per min. Each value represents 2 experiments with duplicate determinations per experiment. S.D. for each figure was less than 18%.

To correlate the epidermal enzyme activity and ^{3}H-PB binding to DNA *in vivo* with benzo(a)pyrene tumorigenesis, we studied the skin tumor incidence initiated with BP in the presence or absence of the epoxide hydratase inhibitor TCPO. The experimental protocol

Table 2. Binding of (^{3}H) benzo(a)pyrene to DNA of skin in pretreated with BP and TCPO mice

Compounds (nmole)	Binding of (^{3}H) BP to DNA of skin μmole per mole P*
BP (100)	1.42±0.16
BP (100)+TCPO (2000)	1.56±0.18

The binding level to DNA was measured 24 hr after application of (^{3}H) BP and TCPO. The binding level at 0°C was 0.36±0.12. The similar results concerning the enzyme activity and the binding level were obtained also when the mice were treated two times weekly.
*Each value represents 2 experiments with duplicate determinations per experiment.

was described in Materials and Methods. The experiment was terminated 50 weeks after the application of the initiating dose of BP and the results are shown in Fig. 2.

The tumors obtained were papillomas, and were included in the results after they reached 1 mm in diameter. Their number and size increased regularly until the 30th week. At this time, some mice of the group initiated with 100 nmole BP once and most of the mice of the group initiated with 100 nmole BP twice are bearing several tumors. After the 30th week, some of the papillomas fell off and the total number of papillomas decrease slightly. The malignant transformation of papillomas, accompanied by ulceration, is a late phenomenon and is not achieved at 50 weeks, so that these tumors are not taken into account in the present results.

No tumor formation in the control group was observed. TCPO alone, in the present experimental conditions, had no effect on normal skin morphology and did not produce the morphologic changes characteristic of a promoter [17]. Skin tumors started to appear about 15 weeks after initiation of the mice at the same time in all groups initiated with BP with or without TCPO. Skin tumor incidence in the mice initiated with BP once or twice was not significantly different, but rather higher as that in the groups that received the initiator with the epoxide hydratase inhibitor TCPO. These results are different from those obtained by Berry and others [16] who found that at 28 weeks after initiation TCPO was effective in increasing the skin tumor initiating ability of BP in CD-1 female mice which had received 10 μmole of TCPO 5 min before initiation.

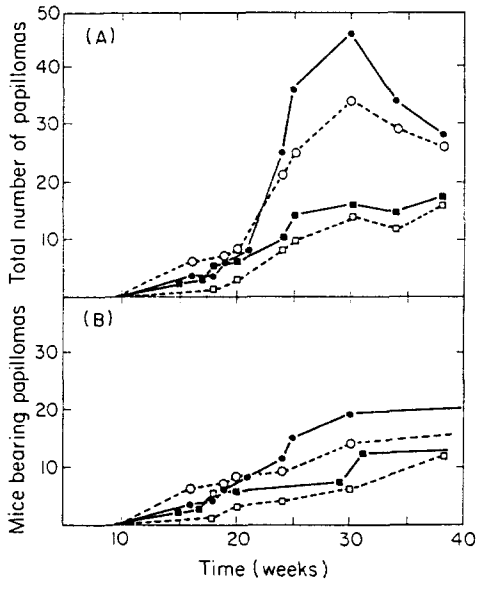

Fig. 2. Skin tumors in mice treated with BP and TCPO. Groups of 25 male mice were used for each agent. A single dose of the initiator (100 nmole) was applied as a solution in acetone (0.2 ml) to the dorsal skin without or in presence of 2000 nmole TCPO.
■——■ 100 nmole *BP*.
□——□ 100 nmole *BP+TCPO*.
●——● 100 nmole *BP twice*.
○——○ 100 nmole *BP+TCPO twice*.

DISCUSSION

The covalent binding of chemical carcinogens to cellular macromolecules, and in particular to nucleic acids, is considered to be an essential aspect in their action [23]. Many carcinogens, including polycyclic aromatic hydrocarbons, require metabolic activation before covalent binding occurs. Arene oxides have been proposed as the reactive intermediates for the polycyclic aromatic hydrocarbons, and evidence has been presented to support this theory [8].

In 1973 Borgen *et al.* [10] showed that metabolic activation of (±) *trans*-7,8-hydroxy-7,8-dihydrobenzo(a)pyrene (BP 7,8-dihydrodiol) by liver microsomes resulted in a much greater binding to added DNA than metabolically activated BP, 3-hydroxybenzo(a)pyrene, BP 4,5-dihydrodiol, or BP 9,10-dihydrodiol. The reactive metabolite involved in the binding to DNA was a *trans*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro BP (BP 7,8-diol-9,10-epoxide) [12]. The nucleoside-hydrocarbon binding profile obtained with BP 7,8-diol-9,10-epoxide, but not with the K-region BP 4,5-oxide, correlated with the nucleoside binding pattern obtained after the metabolic activation of BP by rat liver microsomes [24]. Both stereoisomers of

this diol epoxide, which are enzymatically formed from BP 7,8-dihydrodiol [25], were highly mutagenic to bacterial amd mammalian cells [26, 27] suggesting that a BP 7,8-diol-9,10-epoxide might be an ultimate carcinogenic metabolite of BP.

There is evidence that the diolepoxide (7,8-dihydro-7,8-dihydroxy-benzo(a)pyrene-9,10-oxide) rather than the K-region oxide is the major metabolite involved in nucleic acid binding *in vivo* and the ultimate carcinogenic form of BP. More recently, it has been demonstrated that benzo(a)pyrene 7,8-dihydrodiol is more carcinogenic than benzo(a)pyrene in newborn mice [28].

The metabolic formation of BP diols and diolepoxides from BP emerges from the following pathways: the microsomal NADPH-dependent monooxygenase system (particularly AHH) oxidized BP to BP epoxides which are hydrated to BP dihydrodiols by microsomal epoxide hydratase. The cytochrome P-450 containing system is also required for the epoxidation of BP-diols to dihydrodiols epoxides.

Our goal was to see what would be the effect on skin tumorogenesis if we interfered in the hydration of BP oxides with a potent inhibitor of epoxide hydratase, 1,1,1-trichloropropene oxide (TCPO).

The addition *in vitro* of TCPO at equimolar and higher concentrations to an incubation medium containing BP inhibited the formation of BP diol in the presence of liver nuclei [4, 29] and microsomes [14]. Under the same conditions TCPO increased the NADPH-dependent *in vitro* binding of BP to DNA [4, 14, 17] presumably by increasing the effective concentration of metabolically active epoxide(s). With microsomes it was more difficult to reproduce the correct intracellular concentrations and circumstances of all the necessary enzymes and cofactors.

In contrast to observations with hepatic microsomes [14, 17], nuclei [30] and skin microsomes (Fig. 1), Berry *et al.* [16] observed a lack of stimulation of BP binding to DNA by TCPO when using epidermal homogenates. Little or no accumulation of dihydrodiols occurred while studying the HPLC profile of metabolites after incubation of BP with epidermal homogenates [18]. The studies showed that BP-7,8-diol is a more potent initiator of skin tumorigenesis than other BP metabolites [31]. The greater initiation ability of the BP-7,8-diol is related to its further biotransformation to a diolepoxide, which is considered to be the ultimate metabolite of BP. The low initiating ability of BP-7,8-epoxide and BP-7,8-diol-9,10-epoxide is probably due to their high reactivity: they react with the nearest nucleophiles before reaching the target area. If TCPO, when applied to mouse skin, inhibits epoxide hydratase and the formation of BP-7,8-diol as occurs in the *in vitro* studies with epidermal homogenates [16], the formation of tumors should decrease, that is if the BP-7,8-diol-9,10-epoxide is the "ultimate" BP carcinogen. The results of Berry *et al.* [16, 18] which show an increase of tumor formation support the concept that other BP epoxides contribute to the carcinogenic activity of BP. However, further studies are needed to explain the lack of BP diols and increasing tumor-initiating ability [16] with the conclusion that BP-7,8-diol is the proximate metabolite [28] of BP and its tumor initiating activity [31].

Our results (Table 2) show no increase after 24 hr in the binding level *in vivo* in the presence of TCPO and no effect on tumorigenesis (Fig. 2). The results can be explained first by the low levels of epoxide hydratase in the skin which is about ten times less than in liver. Owing to the low levels of epoxide hydratase activity, the few epoxides formed, which can be prevented by the epoxide hydratase inhibitor from metabolizing to dihydrodiols, cannot reach the steady-state level for an increase of BP binding to DNA. It is also possible that these epoxides through catalysis are trapped by glutathione in the skin spontaneously or by glutathione-S-epoxide transferase. TCPO is a good inhibitor of epoxide hydratase, but it is also a good electrophyle and thus can react with nucleophiles from the skin. So, very soon after its application it is eliminated and does not interfere any more with BP metabolism.

Burki *et al.* [17] reported that topical administration of TCPO with 3-MC to the skin of mice markedly stimulated 3-MC-induced tumor formation. Berry *et al.* [16] revealed that TCPO (10 μmole per mouse) was effective in increasing the skin tumor initiating ability of BP, while our results (Fig. 2) show that TCPO (2 μmole per mouse) did not have any effect on the tumor initiating ability of BP at 50 weeks after application of benzo(a)pyrene. The difference between both data could be explained by the use of different strains of mice (e.g., C57BL6 could be more sensitive than Swiss mice), by a secondary effect of TCPO related to its electrophilic character, perhaps as allergenic or irritating agent or as trapping agent for cellular

glutathion which act as secondary protector against BaP oxides. The very minor difference in experimental protocol seems insufficient to explain the contradictory results of both experiments.

TCPO has also been shown to inhibit the arylhydroxylase at high concentration and would have been expected to decrease the initiating ability of BP. Our experiment and those of Berry *et al.* [16] do not show such a decrease. In opposition to our expectation, TCPO used *in vivo* is not able to suppress the BP initiating activity, and depending upon the strain of mice and timing, may have some favorable action on tumorigenesis in this case.

The problems stress the point that this compound can be used only with great difficulty as a tool in understanding the metabolic activation of polycyclic aromatic hydrocarbons.

Further experiments are needed to determine more precisely the effect of inhibitors of epoxide hydratase on BP tumor initiation. The topical application of TCPO on the skin did not help to determine the role of epoxide hydratase in BP skin tumor initiation.

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