

# Tamoxifen Suppresses Tumor Promoter-induced Hydrogen Peroxide Formation by Human Neutrophils<sup>1</sup>

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## ABSTRACT

*Trans*-tamoxifen (TAM) has been used successfully in therapy for estrogen-dependent human breast tumors and prevention of their recurrence. The mechanism of this prevention was thought to be due to the interference of TAM with estrogen promotion. TAM has a wider anticarcinogenic action that is similar to other chemopreventive agents in that it suppresses tumor promotion in 2-stage carcinogenesis by interfering with the action of protein kinase C. We report that TAM (5  $\mu$ M) totally inhibits hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) formation by 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-treated human neutrophils. Interestingly,  $\beta$ -estradiol (10  $\mu$ M) also slightly inhibits the oxidative burst of neutrophils. Pretreatment of neutrophils with varying amounts of TAM and  $\beta$ -estradiol caused additive inhibition of H<sub>2</sub>O<sub>2</sub> formation by the 2 agents. 4-Hydroxy-tamoxifen, a metabolite with the highest affinity for the estrogen receptor, was only as inhibitory as  $\beta$ -estradiol. Other derivatives (*cis*-, *N*-desmethyl-, and *N*-desdimethyl-tamoxifen) with low biological activities had a smaller effect on H<sub>2</sub>O<sub>2</sub> formation. TPA-treated neutrophils were shown to contain 5-hydroxymethyl uracil (HMU). TAM prevented the TPA-induced formation of HMU in other cells. Like TPA, dietary fat, which is a risk factor for breast cancer, induces formation of HMU in the DNA of human white blood cells. TAM may suppress the dietary fat-induced HMU in the same manner as it does in TPA-induced neutrophils.

## INTRODUCTION

TAM,<sup>3</sup> an antiestrogenic drug, is a successful therapeutic agent in human breast cancer. Studies with estrogen-receptor-positive breast cancer cell lines show that TAM exerts an antiproliferative effect, presumably by competitive interactions with the estrogen receptors that cause suppression of the mitotic action of estrogen (1-4). Recent reports also demonstrate that TAM exerts antiproliferative effects on estrogen receptor-negative breast cancer cells (5, 6), implying that suppression of breast cancer by TAM may include additional factors that are unrelated to competition with estrogen.

In this paper, we report that TAM suppresses hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) formation by intact human neutrophils activated with the tumor promoter TPA and with arachidonic acid. The H<sub>2</sub>O<sub>2</sub> induction was completely inhibited by TAM, a characteristic of many chemopreventive agents, including protease inhibitors, retinoids, sarcophytols, and (-)-epigallocatechin gallate (7-13). TPA induces the oxidative burst in mouse skin phagocytic cells contributing to tumor promotion (14-17). This results in H<sub>2</sub>O<sub>2</sub> production (16), which was shown to be capable

of malignant transformation of cells *in vitro* (18). Moreover, H<sub>2</sub>O<sub>2</sub> itself was shown to contribute to tumor promotion (19, 20). Dietary fat recently was found to cause *in vivo* oxidative damage to DNA bases. Djuric *et al.* (21) showed that women on high-fat diets have a significantly higher ratio of HMU to thymine in their white blood cells than do women on low-fat diets (21). HMU is a product of oxidation of the thymine moiety in DNA, which is formed by reactive oxygen species such as H<sub>2</sub>O<sub>2</sub> (16, 22). The contribution of fat in the diet to the increased rate of breast cancer occurrence in the world population was shown by epidemiological studies carried out by Carroll (23). The lowest rates of breast cancer occurrence were noted in Thailand, where the dominant food staples are rice and soybeans. The highest occurrence was in The Netherlands, where meat and dairy products are the major foods consumed (24). The increased content of HMU in the white blood cell DNA of women on a high-fat diet points to this diet as being the cause of the increased formation of H<sub>2</sub>O<sub>2</sub> and oxyradicals (21). The observation that TAM suppresses H<sub>2</sub>O<sub>2</sub> production and the consequent 5-hydroxymethyl-2'-deoxyuridine formation by cells<sup>4</sup> provides a novel mechanism of its antitumor activity, which is preventing oxidation of DNA bases induced by tumor promoters, including those present in the diet.

## MATERIALS AND METHODS

**Reagents.** TAM citrate,  $\beta$ -estradiol, dextran, phenol red, horseradish peroxidase, catalase, and arachidonic acid were purchased from Sigma. 4-Hydroxy-, *N*-desdimethyl-, *N*-desmethyl-, and *cis*-tamoxifen were kind gifts from Dr. Catherine O'Brian (University of Texas M. D. Anderson Cancer Center, Houston, TX) and from ICI Pharmaceutical (Cheshire, England). Antiestrogen ICI 164,384 was obtained from ICI Pharmaceutical.

**Polymorphonuclear Leukocyte Preparation.** All experiments were performed using neutrophils isolated from blood obtained from consenting healthy volunteers. The blood was drawn into heparinized vacutainer tubes (Becton Dickinson, Lincoln Park, NJ), using standard venipuncture techniques. The neutrophils were isolated using the method described by Frenkel *et al.* (25). This procedure involves dextran sedimentation of erythrocytes (40 min), in which blood is mixed with 6% dextran in 0.15 M NaCl at a 3:1 ratio in a plastic syringe. The resulting buffy coat was collected and centrifuged (Beckman model GPR) at 1500 rpm for 10 min. Contaminating erythrocytes were removed by lysis with a NH<sub>4</sub>Cl solution, containing 0.15 M NH<sub>4</sub>Cl and 0.1 M Tris·HCl, pH 7.2. The neutrophils were isolated and resuspended in a balanced salt solution (137 mM NaCl, 5 mM KCl, 8.5 mM Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub>, 0.8 mM MgSO<sub>4</sub>, 5 mM glucose, pH 7.4) at a ratio of 1 ml of buffer per 10 ml of blood. A hematocytometer count was performed and the cells were adjusted to 2.5  $\times$  10<sup>5</sup> neutrophils/ml for each assay, based on the results of Wright's stain of the peripheral blood. All procedures were carried out in polypropylene tubes, and the prepared neutrophils were used within 4-5 h.

**H<sub>2</sub>O<sub>2</sub> Assay.** H<sub>2</sub>O<sub>2</sub> assays were performed in triplicate using the method based on the horseradish peroxidase-dependent oxidation of phenol red, which was determined by its increased absorbance at 610 nm (16, 26). The oxidative burst was elicited by the addition of 25 nM

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<sup>3</sup> The abbreviations used are: TAM, *trans*-tamoxifen; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; HMU, 5-hydroxymethyl uracil; DHEA, dehydroepiandrosterone.

<sup>4</sup> R. Bhimani and K. Frenkel, unpublished data.

TPA to the neutrophils. Incubation of TPA-treated neutrophils in polypropylene tubes at 37°C for 30 min resulted in H<sub>2</sub>O<sub>2</sub> formation. H<sub>2</sub>O<sub>2</sub> formation-inhibiting agents were added to neutrophils prior to TPA and incubated at 37°C for 15 min, followed by TPA and continued incubation for an additional 30 min. The reactions were terminated by addition of 10 μl of 5 mg/ml catalase followed by changing the pH with 1 M NaOH to about 10.5. The changes in absorbance were determined spectrophotometrically (Gilford Response II) in 1-ml cuvettes (Starstedt) at 610 nm.

## RESULTS

Complete inhibition of H<sub>2</sub>O<sub>2</sub> formation by TPA-stimulated neutrophils was achieved with TAM at a concentration of 5 μM (Fig. 1). β-Estradiol suppressed H<sub>2</sub>O<sub>2</sub> formation to a much lower extent (less than 40% at 10 μM). The latter inhibition was similar to the action of 4-hydroxy-tamoxifen, the metabolite of TAM that has the highest affinity for the estrogen receptor. The TAM derivatives with low antiestrogen biological activity (*cis*-, *N*-desmethyl-, and *N*-desdimethyl-tamoxifen) were not too effective in suppressing H<sub>2</sub>O<sub>2</sub> formation.

When β-estradiol and TAM were added at the same time, their effects on diminishing H<sub>2</sub>O<sub>2</sub> induction were additive. The additivity suggests that these 2 agents act on different sites to exert the suppression of H<sub>2</sub>O<sub>2</sub> induction. Table 1 shows the effect of different concentrations of β-estradiol added together with 1 μM TAM. The effect of 1 μM β-estradiol, added together with the different concentrations of TAM on TPA-mediated H<sub>2</sub>O<sub>2</sub> formation, is shown to be again additive rather than synergistic or competitive (Fig. 2).

The antiestrogen ICI 164,384 acted in a manner that was opposite to those of TAM and β-estradiol in that it increased TPA-induced H<sub>2</sub>O<sub>2</sub> production by human neutrophils. TAM (2.5 μM) suppressed it, while β-estradiol failed to suppress it. These results demonstrate separate types of action by β-estradiol and TAM on lowering H<sub>2</sub>O<sub>2</sub> induction (Fig. 3). The action of ICI 164,384 recently was shown to be due to its decreasing the stability of the estrogen receptor (27).

## DISCUSSION

The identification of chemopreventive agents by Wattenberg (28) offered a new opportunity to design ways of preventing human cancer. A general characteristic of many chemopreven-

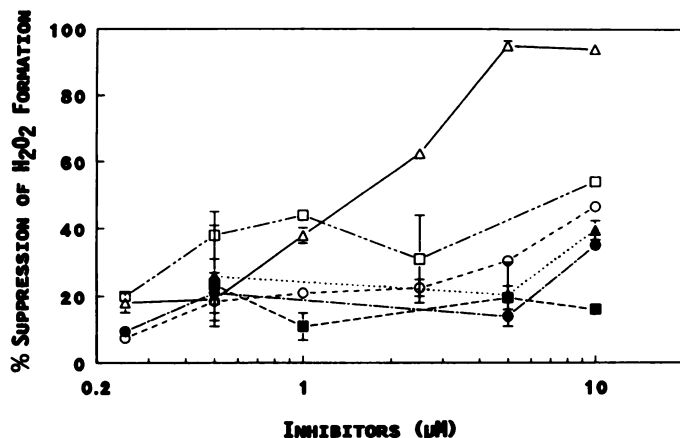


Fig. 1. Effects of TAM (Δ), 4-hydroxy-tamoxifen (□), *N*-desdimethyl-tamoxifen (▲), *N*-desmethyl-tamoxifen (●), *cis*-tamoxifen (■), and β-estradiol (○) on the inhibition of H<sub>2</sub>O<sub>2</sub> production by 25 nM TPA-stimulated human neutrophils. Bars, mean ± SE. Absence of error bars, SE within the symbols.

Table 1 Combined effect of TAM and β-estradiol on suppressing H<sub>2</sub>O<sub>2</sub> induction

The additive action on the inhibition of H<sub>2</sub>O<sub>2</sub> formation by 25 nM TPA-stimulated human neutrophils when TAM and β-estradiol are added either singly<sup>a</sup> or as mixtures.<sup>b</sup>

Agent	Conc. (μM)	% inhibition		
		Agent alone	Calculated additive effect <sup>c</sup>	Mixture of 1 μM TAM and β-estradiol <sup>b</sup>
TAM	1	38		
β-Estradiol	1	20	58	65
	5	30	68	69
	10	45	83	82

<sup>a</sup> Agent alone.

<sup>b</sup> Experimental percent inhibition caused by adding 1 μM TAM and β-estradiol (1, 5, and 10 μM, respectively) together to polymorphonuclear leukocytes.

<sup>c</sup> Sum of percent inhibition calculated by adding that mediated by 1 μM TAM to those induced by β-estradiol (1, 5, and 10 μM, respectively).

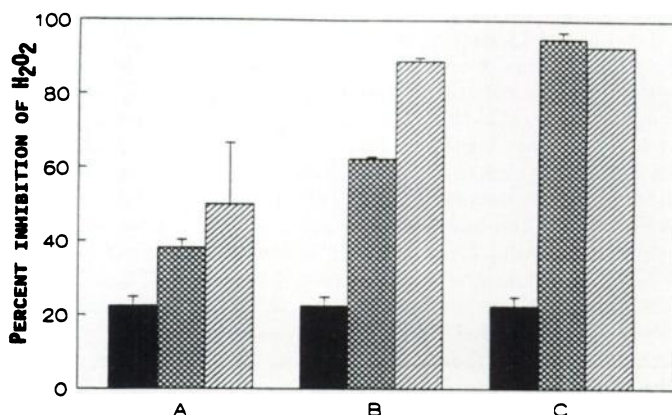


Fig. 2. Effects of increasing concentrations of TAM on the action of 1 μM β-estradiol on human neutrophils activated by 25 nM TPA. Reaction conditions are the same as in Fig. 1 and "Materials and Methods." A, 1 μM β-estradiol (■), 1 μM TAM (□), and 1 μM each of β-estradiol and TAM (▨); B, 1 μM β-estradiol (■), 2.5 μM TAM (□), and 1 μM β-estradiol, and 2.5 μM TAM (▨); C, 1 μM β-estradiol (■), 5 μM TAM (□), and 1 μM β-estradiol, and 5 μM TAM (▨). Data are mean ± SE.

tive agents is their interference with oxidative processes. *Trans*-retinoic acid, sarcophytol A, protease inhibitors, and (–) epigallocatechin gallate are examples of chemopreventive agents that are capable of suppressing animal tumors and formation of H<sub>2</sub>O<sub>2</sub> by TPA-treated human neutrophils (7–13, 29–31). The demonstration that TAM is perhaps the most effective suppressor of H<sub>2</sub>O<sub>2</sub> production by TPA-stimulated neutrophils is unexpected, since its action was thought to be due entirely to the interference with β-estradiol-mediated enhancement of tumor promotion. TAM, in common with other chemopreventive agents, blocks 2-stage carcinogenesis in mouse skin by inhibiting the activation of protein kinase C (32, 33). HMU and 8-hydroxyguanine formations were noted in TPA-treated neutrophils and in coincubated cells (9, 31, 34–36). DHEA is a hormone that suppresses breast, colon, and lung cancers in mice, and inhibits TPA-induced formation of superoxide anion by human neutrophils (37). Recently it was shown that the levels of DHEA are significantly increased in female adolescent Seventh-Day Adventists, whose diets are vegetarian (38). DHEA has some of the characteristics of TAM in that it suppresses breast cancer in animals and induction of oxyradicals by neutrophils. The recent epidemiological data suggest that it also may be responsible for the suppression of many cancers in vegetarian populations. It will be of interest to study whether TAM is also capable of suppressing a variety of tumors, including colon and lung. It is tempting to ascribe these oxidative

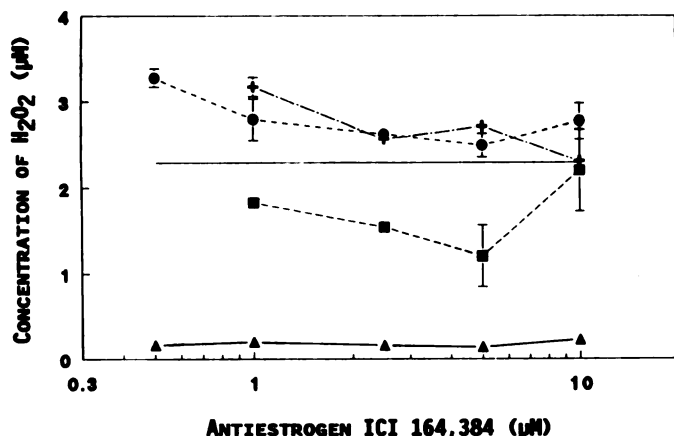


Fig. 3. Effects of tamoxifen and  $\beta$ -estradiol on the antiestrogen ICI 164,384-mediated increase of  $\text{H}_2\text{O}_2$  production by 25 nM TPA-stimulated human neutrophils. Reaction conditions are the same as in "Materials and Methods." (—), TPA only; (Δ), antiestrogen only; (●), antiestrogen + 25 nM TPA; (■), antiestrogen + 25 nM TPA + 2.5  $\mu\text{M}$   $\beta$ -TAM; +, antiestrogen + 25 nM TPA + 5  $\mu\text{M}$   $\beta$ -estradiol. Bars, mean  $\pm$  SE. Absence of error bars, SE within the symbols.

DNA modifications as being the basis for the carcinogenic action of the oxidative burst, since the formation of oxidized bases was noted also in human breast tumors (39). TAM could be effective in suppressing the formation of oxidized bases in breast tissue.

The antioxidative properties of TAM in suppressing  $\text{H}_2\text{O}_2$  formation were demonstrated also in sea urchin eggs, which (like neutrophils) produce  $\text{H}_2\text{O}_2$  when the egg is activated by sperms. This production of  $\text{H}_2\text{O}_2$  leads to rapid formation of a fertilization envelope, which prevents the entry of other sperms that would cause irregular polyspermic division (40). When TAM or  $\beta$ -estradiol is added before fertilization, they suppress the sperm-mediated induction of  $\text{H}_2\text{O}_2$  (41). This prevents the formation of the fertilization envelope and allows entry of the other sperms that cause polyspermic division. The pure antiestrogen ICI 164,384 acts in an opposite manner, forming a  $\text{H}_2\text{O}_2$ -induced fertilization envelope before fertilization, thus preventing subsequent fertilization (41).

ICI 164,384 was shown to completely block the stimulatory activity of exogenous  $\beta$ -estradiol in immature ovariectomized adult mice. However, it was less effective than TAM in the suppression of 7,12-dimethylbenz(a)anthracene-induced mammary tumors in intact or ovariectomized mice (42, 43). Fat in the diet of women can be considered a tumor promoter whose action is similar to that of TPA in the experimental 2-stage carcinogenesis model. The promotion by fat may be mediated by fatty acid metabolites, such as arachidonic acid, which mimics the action of TPA in inducing  $\text{H}_2\text{O}_2$  formation in human neutrophils (44). When TAM (1  $\mu\text{M}$ ) was added to arachidonic acid (20  $\mu\text{M}$ )-stimulated human neutrophils, it inhibited  $\text{H}_2\text{O}_2$  production by 39%, which is similar to the 38% inhibition by TPA-stimulated human neutrophils (Table 1). Further work is in progress with other fat metabolites.

The actions of both TPA and dietary fat are similar in that they cause the formation of the oxidized thymine HMU in DNA (21, 31, 34). TAM suppresses the formation of HMU and 8-hydroxyguanine in epidermal cells of TPA-treated SENCAR mouse skin (13). In the interesting studies of the effects of high- and low-fat diets on the levels of oxidative damage to DNA in human peripheral nucleated blood cells, Djuric *et al.* (21) noted, in women on high-fat diets, a 3-fold increase of HMU levels. They also observed that the mean DNA damage level of pre-

menopausal women was slightly lower than that of postmenopausal women. This effect could be due to higher estrogen or DHEA production in premenopausal women, which suppresses the formation of  $\text{H}_2\text{O}_2$  that leads to the lower extent of DNA oxidation.

Chemopreventive substances are a large group of structurally varied agents that interfere with the activation of carcinogens or the promotion and progression of cancer, perhaps by suppressing the formation of  $\text{H}_2\text{O}_2$  and oxyradicals (28, 31). The formation of oxidized DNA bases by these active oxygen species is similar to that of ionizing radiation, which generates oxyradicals that can act as a cancer initiator as well as a promoter (31, 45). The formation of the oxidized DNA bases may lead to mutation, a characteristic of initiation of cancer by oxyradicals and other carcinogens (31, 46). Therefore, preventing the formation of these oxidized DNA bases may block the process of tumor formation. TAM inhibits neutrophil infiltration, as well as formation of  $\text{H}_2\text{O}_2$  and oxidized DNA bases in TPA-treated mouse skin (13, 31). It also suppresses formation of  $\text{H}_2\text{O}_2$  by activated human neutrophils when used at a concentration that is comparable to that present in the plasma of patients who received TAM. Hence, TAM may also be capable of preventing initiation as well as promotion of breast cancer. Further epidemiological studies of the role of TAM in the prevention of breast cancer will help to establish whether it also can decrease the initiation stages of breast cancer.

Perhaps the most surprising finding, that the suppression of  $\text{H}_2\text{O}_2$  induction is enhanced when  $\beta$ -estradiol and TAM are used together, suggests that their combined use may be more effective in preventing cancer than TAM alone. This may extend the usefulness of TAM to prevent cancer in premenopausal women and may give the opportunity to use  $\beta$ -estradiol plus TAM in postmenopausal women with the desirable opportunity of preventing cancer.

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